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The purpose of this project is to 1) construct novel bispecific antibodies that can redirect the activity of T cells against a tumor and 2) develop *in vivo* animal models that more closely resemble human cancer, for evaluating these agents. In the past year we have focused on the design and testing of antibody-based agents that will increase the activity of T cells and that will sustain this activity against a tumor. This aspect of the project has made use of novel bispecific folate/antibody conjugates that target the high affinity folate receptors on tumor cells. We have shown that combinations of folate/anti-TCR antibodies and folate/anti-CD28 antibodies are synergistic in stimulating T cell activity *in vitro*. The induced activity was completely dependent on the presence of the tumor, suggesting that there will be minimal side effects due to systemic cytokine release by non-specific T cell activating agents. In a complementary approach, monovalent forms of an anti-CTLA-4 antibody (Fab and scFv) were produced and shown to further sustain the activity of T cells *in vitro*. Finally, folate/anti-TCR antibody agents were tested in an *in vivo* animal model of an endogenously arising tumor of the choroid plexus. Treated animals had extensive T cell infiltration into the site of the tumor and significant increases in survival. Further studies will explore the use of the T cell stimulating agents described above, in this animal model.

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INTRODUCTION
(Adapted in part from original proposal)

Successful treatment of breast cancer requires the identification of specific targets for the rational design of therapeutic agents. One such target, described several years ago, is the oncogene product erbB-2. This protein is expressed on the surface of tumor cells in approximately 30% of women with the poorest prognosis for survival (1). A number of investigators have now begun to use antibodies specific for erbB-2 as possible therapeutic agents (2-13). In fact, the past year has seen one of the significant advances in breast cancer treatment with successful clinical trials of the Genentech anti-erb-B2 antibody "Herceptin"(14, 15).

Other anti-erb-B2 agents that are at earlier stages of development include immunotoxins, radioimmunoconjugates, and bispecific antibodies. The latter antibodies are intended to mediate effects by directing the lysis of tumor cells through T cell effectors. One of the predicted advantages of bispecific antibodies is that they should not have side effects associated with delivery of toxins or isotopes. Despite their promise and emergence into clinical trials, there are many questions that need to be addressed before optimal uses, with minimal side effects, of bispecific antibodies can be realized. For example, genetic engineering now provides a method for constructing smaller, potentially more stable, antibodies. Are these antibodies likely to be more effective than conventional intact antibodies? Can a system that uses a patients own immune cells be developed so that *ex vivo* activation of effector cells is not needed? This would obviously allow the treatment of a much larger patient base than would be possible if effector cells must be cultured for every patient.

It would clearly be useful to have a system that could provide answers to these questions in order to design the most effective clinical trials of bispecific antibodies. There has been no animal model developed that can evaluate all of these issues using human breast cancer cells. The purpose

of this project has been twofold. First, to construct novel bispecific antibodies that will show optimal tumor targeting potential. Second, to develop *in vivo* animal models that will most closely resemble human cancer and its treatment. The animal models should allow the testing of the bispecific agents in comparison with other conventional bispecific antibodies. There are many potential therapeutic regimens that will need to be evaluated. To do so, an animal model that will not require introduction of human effector cells and that will most resemble the situation that will be encountered in the human disease will be developed.

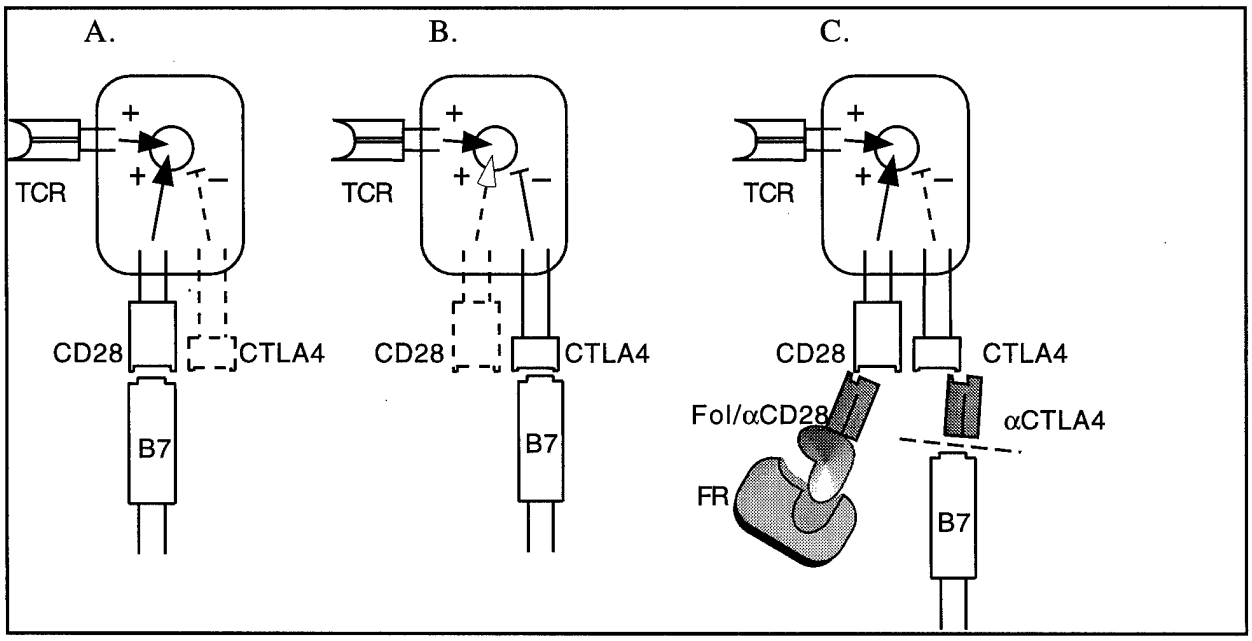
The original four specific aims of this project were: 1) Construct and characterize a single-chain bispecific antibody (anti-erbB-2/anti-TCR, called $scFv_2$); 2) To use a simple screening method to search for agents that increase the sensitivity of erbB-2^+ breast cancer cell lines to lysis by CTL; 3) To develop an *in vivo* model for targeting transplanted human breast cancer cells using immunodeficient, TCR transgenic mice (TCR/recombination-activating-gene knockouts, $RAG^{-/-}$); and 4) To test the *in vivo* effectiveness of various bispecific antibody regimens in the $TCR/RAG^{-/-}$ human xenograft system.

Since the submission of this grant application there have been a number of findings that have impacted the direction of the project. Perhaps chief among these is the basic understanding of how T cells are activated and inactivated (16-19). Understanding these processes are key to the rational development of bispecific antibodies that control and redirect the activity of T cells. It is especially relevant to this project that the activation of T cells is now known to be a complex process involving the triggering of multiple surface molecules (Figure 1, next page). It is clear that at least two signals must be received by the T cell: Signal 1 through the T cell receptor/CD3 complex and Signal 2 through the co-stimulatory molecule CD28 (which binds to the ligand B7).

There has also been considerable interest in the past two years in the molecule CTLA-4, present on the surface of activated T cells. CTLA-4 functions as a negative regulator of T cells (20, 21) and blocking of its interaction with its ligand B7 leads to sustained T cell activity (see Figure 1, next page). In several studies, treatment with anti-CTLA-4 antibodies led to T cell-dependent elimination of the tumors (22-24). Given this complexity, it has become even more apparent to us that the successful application of bispecific antibodies in breast cancer therapy will require the use of defined animal models to evaluate not only the various agents that redirect the activity of T cells to tumors (as described in the original grant application) but to evaluate the various agents that might lead to sustained activity of T cells.

In this report, we show our progress toward this end, focusing on three different goals: **1) Increasing T cell activity with tumor-dependent, bispecific co-stimulatory antibodies; 2) Engineering and characterizing the anti-CTLA-4 antibody 4F10, and showing its use in sustaining T cell activity; and 3) Using bispecific antibodies in the SV40 transgenic tumor model.** Accomplishments toward these goals are provided in the body of this report. This is followed by a list of individual tasks and their status. Because of our increased efforts on these aspects of the project, we have halted our efforts to improve the production and activity of bispecific anti-erbB-2 antibodies. As described in last year's report, these antibodies have received considerable attention from companies that focus on protein engineering. We believe that it would be wiser for our lab to focus on the general principles that will guide the optimal use of these agents in redirecting the activities of T cells against breast cancer.

Figure 1. Diagram of the positive or negative signals induced by multivalent cross-linking of CD28 or CTLA4, respectively, and monovalent inhibition of CTLA4. **A.** Multivalent cross-linking of CD28 through B7 on APC leads to enhanced T cell proliferation. **B.** Multivalent cross-linking of CTLA4 through B7 on APC leads to inhibition of T cell proliferation. **C.** Enhanced signalling through FR-mediated cross-linking of CD28 and inhibition of CTLA4 signals through monovalent anti-CTLA4, as proposed in the studies described here.



BODY

1) Increasing T cell activity with tumor-dependent, bispecific co-stimulatory antibodies.

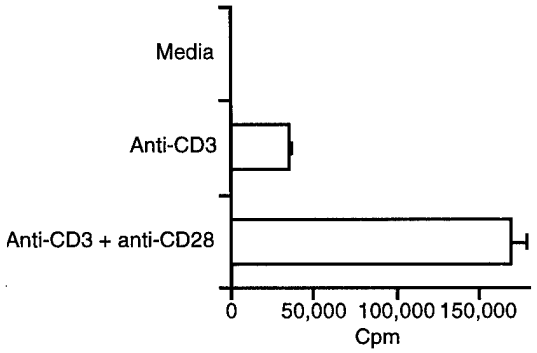
A recent study using anti-erbB-2 linked to the T cell co-stimulatory ligand B7 (see below) illustrates the potential of approaches described in this annual report (25). In the case of using bispecific anti-co stimulatory antibodies, as opposed to the B7-fusion approach, is that the negative signals mediated through the CTLA-4 pathway can be prevented. To explore the use of bispecific co-stimulatory antibodies, we have made use of a simple strategy for preparing conjugates against a tumor antigen. High affinity folate receptors (FR) are present on most ovarian and choroid plexus tumors and on some breast tumors (26-29).

The high affinity of folate for the FR suggested to us that attachment of folate directly to an anti-TCR antibody might efficiently target FR^+ tumor cells for lysis by activated T cells. We reported that such ligand/antibody conjugates have very potent targeting activity without adversely affecting normal cells that express only the reduced folate carrier protein (30, 31). Effective killing was observed with folate conjugates of 145-2C11 (an antibody against CD3), KJ16 (an antibody against Vβ8), and 1B2 (an antibody against the TCR of CTL clone 2C). Lysis of FR^+ tumor cell lines could be detected at concentrations as low as 1 pM.

Effective targeting of tumors with bispecific anti-TCR antibodies requires that T cells be activated. One strategy to effect this activation has been the use of intact anti-CD3 antibody 2C11 IgG (32). This activation can be enhanced in combination with the anti-CD28 antibody 37.51 (33). To test if this combination of agents was effective in our animal models, we have tested both antibodies *in vitro* with two different sources of T cells. In one case, T cells were derived from spleens of the 2C TCR/RAG$^{-/-}$ mice, described in last years report. In the other case, T cells were derived from spleens of normal C57BL/6 mice. Results from both studies showed that anti-CD28 antibody enhanced T cell stimulation in proliferation assays (Figure 2A and data not shown). In addition, anti-CD28 costimulation of T cells enhanced the ability of the effector cells to lyse FR^{+} tumor cells in the presence of the folate/anti-TCR antibodies described above (Figure 2B).

Figure 2. Anti-CD3 plus anti-CD28 synergize in the activation of T cells.
(A) Proliferation. 2C TCR/RAG$^{-/-}$ splenocytes (2 x 10^5 cells/well) were incubated for 48 hrs with anti-CD3 antibody 2C11 (0.1 μg/ml), with or without anti-CD28 antibody 37.51 (5 μg/ml), and then assayed for proliferation by ^{3}H-thymidine incorporation.
(B) Cytotoxicity. C57BL/6 splenocytes were cultured for three days in the presence of media alone, 2C11 (0.1 μg/ml), or 2C11 plus 37.51 (0.01 - 5.0 μg/ml). Cultured cells were then assayed for cytotoxicity of FR^{+}, ^{51}Cr -labeled F2-MTX^{r}A cells in the presence of anti-CD3/folate conjugate (0.15 μg/ml).

A.



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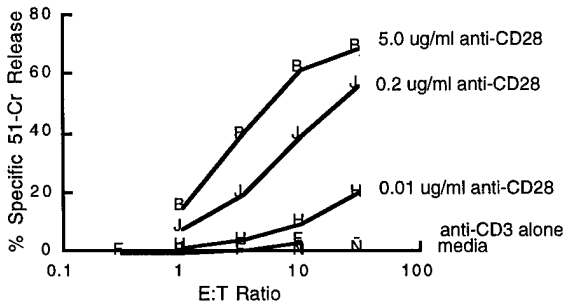
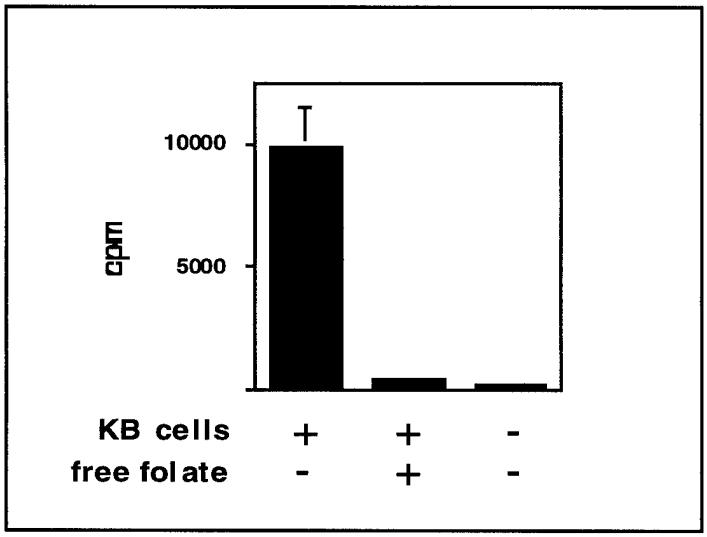


In the induction of both proliferation and cytotoxicity, the effects of 2C11 and 37.51 are presumably dependent on FcR cross-linking. When these effects occur systemically through the general activation of T cells, serious shock-like symptoms can result (34). In order to prevent these effects, it would be useful to generate optimal tumor-dependent T cell activation. We reasoned this might be achieved by attaching folate to Fab fragments of the anti-TCR antibody and the anti-CD28 antibody. In the experiment shown below (Figure 3), folate attached to the anti-TCR antibody KJ16 was capable of stimulating T cell proliferation and this proliferation was totally dependent on the presence of the tumor that express the FR.

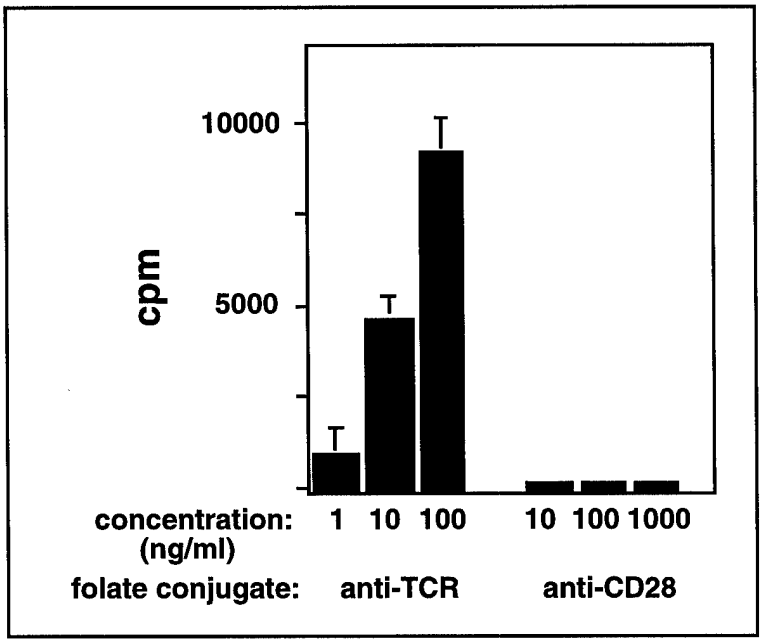
Figure 3. Bispecific folate/anti-TCR Fab conjugates stimulate T cells in tumor-dependent activation. Folate/anti-TCR Fab fragments (KJ16, 10 ng/ml) were incubated with 10^5 spleen cells from a TCR/RAG- mouse (35) for three days at 37C, in the presence (+) or absence (-) of 10^4 KB cells (mitomycin treated, FR+ cells), or in the presence (+) or absence (-) of 10 μM free folate. ^{3}H-thymidine was added for 20 hours and T cell proliferation was measured by the incorporation of cpm.



To test if folate coupled to anti-CD28 Fab fragments was capable of synergizing with this effect, combinations of the folate/anti-TCR and folate/anti-CD28 were added to T cells in the presence of the FR^+ line KB. As shown below, folate/anti-CD28 alone was incapble of stimulation, as predicted from the two-signal mechanism. However, the combination of folate/anti-TCR and folate/anti-CD28 yielded a two-fold enhancement of T cell activation.

Figure 4. Folate/anti-CD28 Fab conjugates co-stimulate T cells in tumor dependent activation. **A.** Folate/anti-TCR Fab fragments and folate/anti-CD28 Fab fragments were incubated separately with 10^5 spleen cells from a TCR/RAG- mouse at the indicated concentrations, as described in Figure 3, in the presence of 10^4 KB cells (mitomycin treated, FR+ cells). **B.** Folate/anti-TCR Fab fragments and folate/anti-CD28 Fab fragments were added together at the indicated concentrations in a proliferation assay, as described above.

A.



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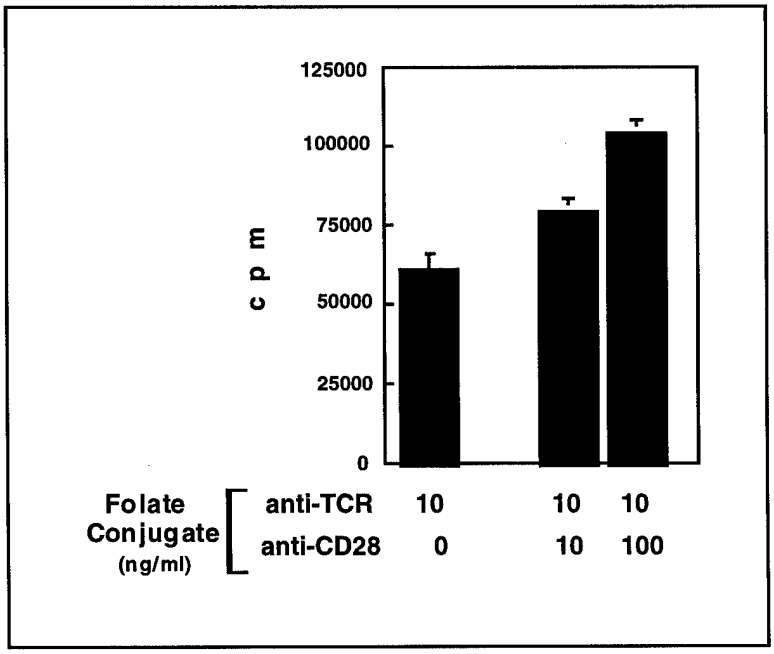
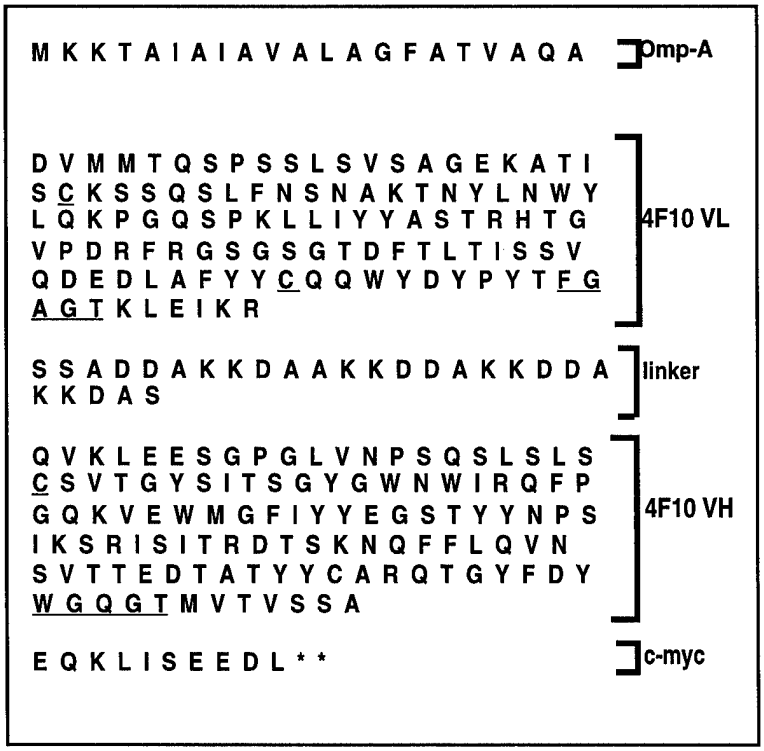


2) Engineering and characterizing the anti-CTLA-4 antibody 4F10, showing its use in sustaining T cell activity.

It has been shown that anti-CTLA4 antibodies are capable of blocking the inactivation of T cells and that they can thereby enhance anti-tumor activity (22-24, 33, 36). However, intact anti-CTLA4 antibodies can also inactivate T cells, if the antibody is presented in multivalent form by FcR bearing cells (37). To eliminate this latter possibility, our lab has begun a collaboration with Jeff Bluestone to engineer monovalent scFv of the anti-CTLA4 antibody 4F10. The monovalent scFv and Fab fragments of the antibody have been used in an attempt to sustain the activity of T cells. In addition, the scFv can now be used to engineer more potent forms of the antibody through directed evolution (a project that will be pursued in the next year).

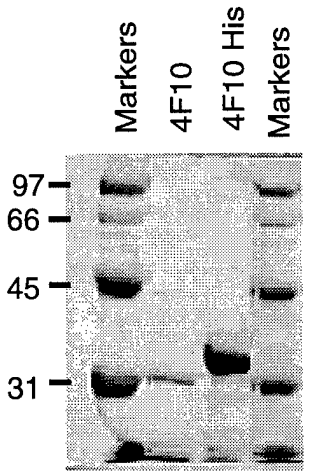
To clone and express the scFv of the anti-CTLA4 antibody 4F10, V_H and V_L genes were amplified from hybridoma cDNA with degenerate primers. V_H, J_H , V_L, and J_L primers were based on the alignment of several published hamster V regions (38, 39). PCR products were cloned into a temperature inducible *E. coli* expression plasmid, behind the ompA signal sequence. This scFv expression vector also contains a ten residue c-*myc* region at the carboxy terminus to allow analysis of total protein expression using anti-c-*myc* antibodies in an ELISA and binding to T cells by flow cytometry. The V_H and V_L sequences were in frame and they conformed to the expected Ig-like domains, incuding invariant cysteine residues and the FGXGT and WGXGT of J_L and J_H regions (Figure 5). In addition, the V_L chain sequence was consistent with amino terminal a. a. sequence (DIMMTQSPSS, where the primer only extended through the Q) obtained from the 4F10 light chain.

Figure 5. Sequence of anti-CTLA-4 scFv-4F10.



Inclusion bodies of the scFv-4F10 were solubilized in 6M guanidine in 100 mM Tris, 2mM EDTA, pH 8, and refolded by dialysis into a Tris/Arginine buffer or diluted 100 fold into a Tris buffer. A 30 kDa scFv protein (Figure 6) was detectable by ELISA and Western blots with anti-c-*myc* antibodies (data not shown). Although both refolding methods yielded detectable protein, the dilution method followed by concentration of the sample was found to yield the most active preparations. Because yields using this method have been uniformly low (100 µg/liter of culture), other refolding conditions are currently being tested. In addition, a 6His tail was cloned at the 3' end of the scFv for purification over a Ni column. Considerably more scFv protein has recently been obtained using this method (Figure 6).

Figure 6. SDS-PAGE gel of the single-chain Fv of anti-CTLA4 antibody 4F10. The scFv 4F10, with or without a hexahistidine tail, was overexpressed in *E. coli*. Cells were lysed by microfluidizing, the isolated inclusion bodies were solubilized in 6M guanidine in 100 mM Tris, 2 mM EDTA, pH 8.0, and refolded by dialysis into Tris buffer containing 0.4 M arginine. After electrophoresis, he gel was stained with Coomasie blue.



Unlabeled Fab fragments from 4F10 and the scFv-4F10 were compared by saturation and competition binding experiments with ^{125}I-labeled Fab fragments. For these experiments, CHO cells transfected with the CTLA4 gene (obtained from Jeff Bluestone) were used in an oil spin assay. As shown in Figure 7, the Fab fragments exhibited a K_D = 98 nM. Partially purified and refolded scFv showed complete inhibition of binding by ^{125}I-Fab fragments. In addition, binding of the crude refolded scFv to CTLA4 transfected CHO cells was detected with anti-c-*myc* antibodies (data not shown). Efforts to purify larger quantities of the scFv-4F10 are under way.

A negative proliferative signal can be elicited by multivalent cross-linking of CTLA-4. This cross-linking can be achieved either by the CTLA4 interaction with B7 on APCs or by the CTLA4 interaction with anti-CTLA4-IgG presented by FcR-bearing cells (see Figure 1). Monovalent anti-CTLA4 (scFv or Fab) can prevent the CTL4:B7 interaction and thereby allow optimal positive signaling generated through CD28 (Figure 1C). We have recently confirmed the hypothesis that a single chain form of anti-CTLA4 will enhance T cell activation, as evidenced in a proliferation assay (Figure 8).

Figure 7. Binding of anti-CTLA4 antibody 4F10 Fab fragments and single-chain Fv fragments to CTLA4.

(A) Binding of ^{125}I-4F10 Fab Fragment. Various concentrations of the iodinated-4F10 Fab fragments were added to Chinese Hamster Ovary (CHO) cells transfected with the CTLA4 gene (CTLA4 CHO). After 30 min on ice, bound ligand was separated from free ligand by centrifugation through dibutyl phthalate/olive oil, and pelleted cells were counted in a gamma counter.

(B) Competitive Binding by scFv 4F10. CTLA4 CHO cells were incubated with ^{125}I-labeled 4F10 Fab fragments and serial dilutions of unlabeled 4F10 Fab fragments or 4F10 scFv fragments for 1 hr on ice. Bound and free were separated by centrifugation of the cells through oil as above. Percentage inhibition was caculated as ((cpm bound with no inhibitor) - (cpm bound with inhibitor)) x 100)/(cpm bound with no inhibitor).

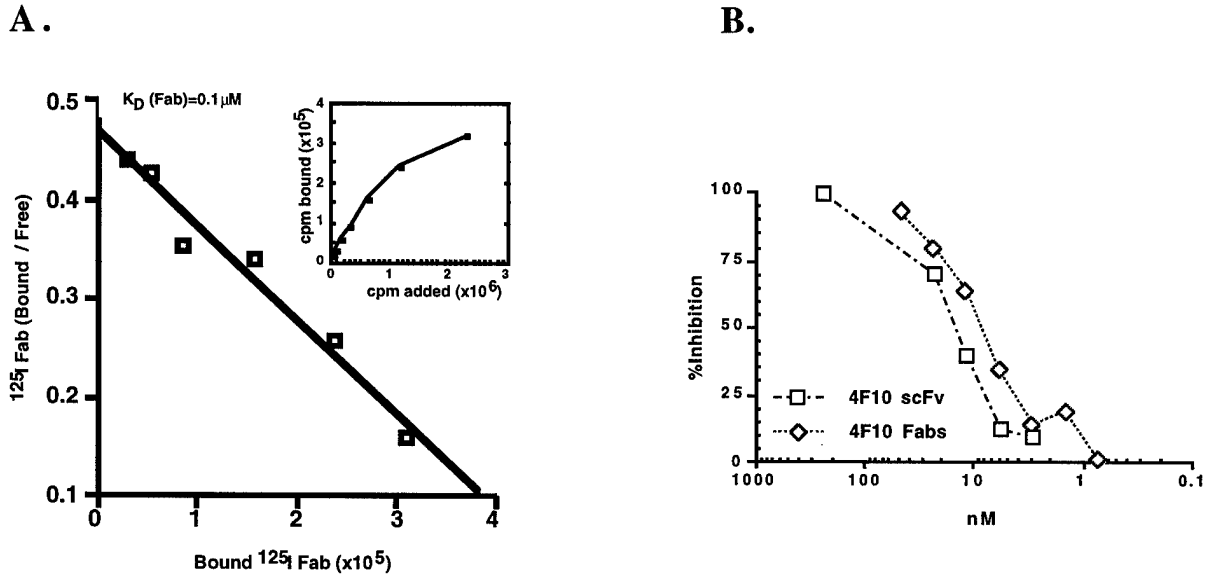
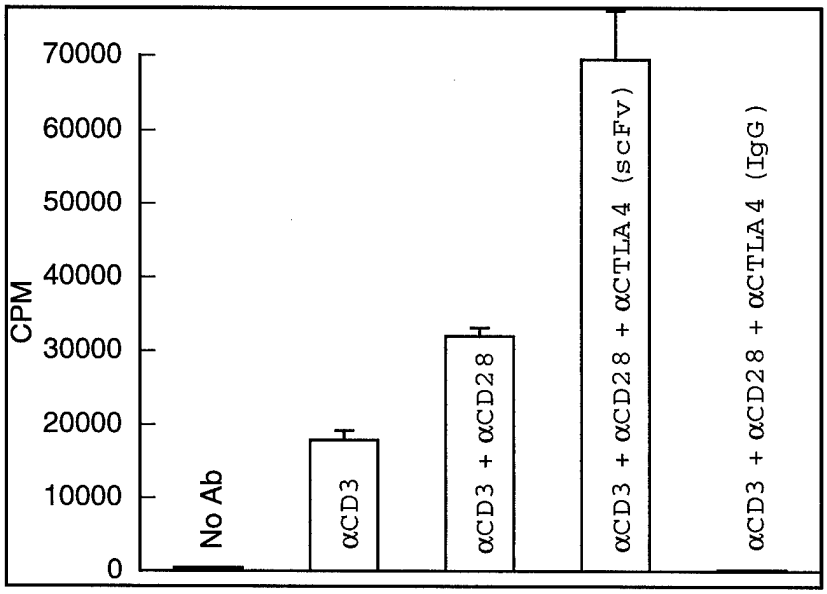


Figure 8 illustrates that anti-CD28 combined with anti-CD3 induces proliferation of a purified T cell preparation from C57 mice. Anti-CTLA4 scFv further increased this proliferation. As noted above, the IgG form of anti-CTLA4 can induce the opposite effect (i.e. reducing T cell proliferation). This latter result is presumed to be due to cross-linking of the anti-CTLA4 by FcR-bearing cells. This finding illustrates the difference between crosslinking and blocking antibodies, and provides the rationale for why it would be counter productive to folate conjugate the anti-CTLA4 scFv antibody.

Figure 8. Enhancement of T cell proliferation with monovalent anti-CTLA-4 antibodies. Splenocytes were isolated using a Variomacs magnetic cell sorter using anti-Thy 1.2, and 2 x 10^5 cells were cultured in 96 well plates for 60 hrs in the presence of indicated antibodies (0.01 µg/ml anti-CD3, 0.1 µg/ml anti-CD28, 10 µg/ml scFV anti-CTLA4, 10µg/ml IgG anti-CTLA4), following by 12 hrs of incorporation of ^{3}H-thymidine. Cpm are means + SEM of triplicate determinations. A titration of antibody concentrations produced similar results (not shown).



3) Using bispecific antibodies in the SV40 transgenic tumor model.

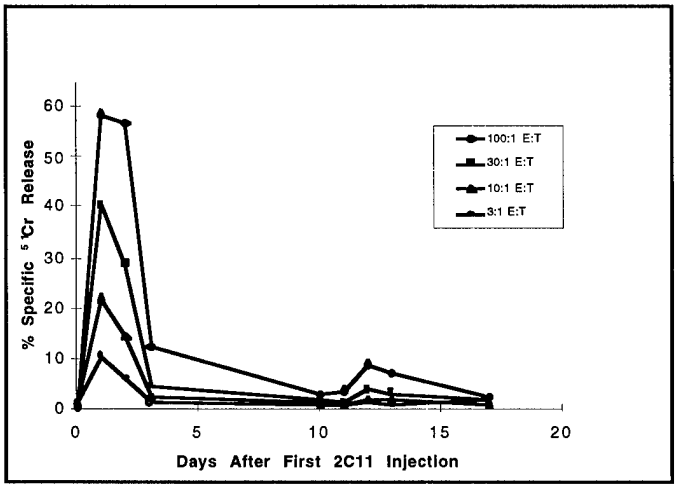
As indicated above, most ovarian tumors and choroid plexus tumors, and some breast tumors express a high-affinity receptor for folate. As a model of an endogenously arising FR$^+$ tumor in an immunocompetent host, we chose SV11 mice that are transgenic for the SV40 large T antigen and develop choroid plexus tumors (40). The etiology of SV11 tumors (i.e. SV40 large T antigen interferes with p53 and pRB) may mimic that of some pediatric brain tumors. SV40 has been isolated from pediatric choroid plexus tumors and ependymomas (41, 42) (the significance of finding SV40 in these tumors and other types of human tumors is still being debated (43). In addition, a high affinity folate receptor has been identified on pediatric choroid plexus tumors and ependymomas (44). The SV11 strain of mice is on a C57BL/6 background and was originally reported to have a mean survival age of 104 days (standard deviation, 12 days)(40, 45). Our colony of SV11 mice, now over 30 generations later, has a nearly identical survival curve, with mean survival times of 100-105 days (for various cohorts).

Because human choroid plexus tumors express high levels of FR, we evaluated endogenously arising brain tumors of the SV40 transgenic mice for the expression the high affinity folate receptor. The FR were characterized by several approaches (e.g. Western Blotting, RT-PCR, immunohistochemistry, and ^{125}I-folate binding assays). In published studies, we found that the tumors express FR with properties that are very similar to the human receptor (46). These properties included its molecular weight (~38 kDa), size of transcripts, and a binding affinity (K_D) for folate of ~ 1 nM. Flow cytometry of FR on the tumor cells and immunohistochemistry indicated that virtually all of the viable cells are FR positive.

In order to evaluate methods for activating T cells *in vivo* in the SV11 mice, preliminary experiments were performed with anti-CD3 2C11 administered to C57BL/6 mice. Because

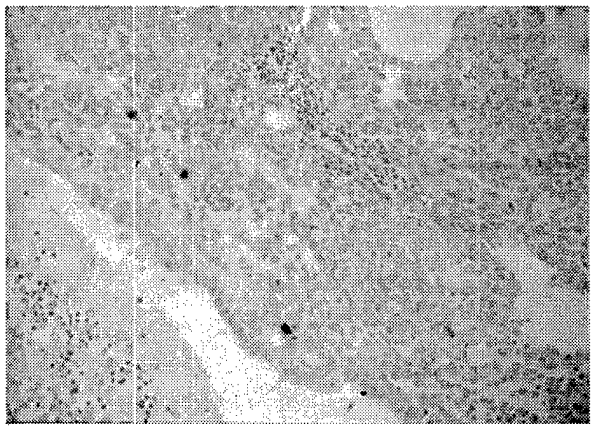
cytotoxicity is the measure most relevant to bispecific therapy, a cytotoxicity assay to directly access the activity of splenic T cells was used. Splenocytes were used *ex vivo*, directly after harvesting without expansion in culture. C57BL/6 mice were injected with various doses of 2C11, sacrificed at time points following injection, and splenocytes were used as effectors. ^{51}Cr-labeled FR$^+$ target cells (F2-MTXrA) were assayed in the presence of bispecific antibody conjugate 2C11/folate. 2C11 was capable of activating splenocytes for about two days but thereafter T cells were non-responsive to restimulation with 2C11 (Figure 9). These observations are consistent with previous measures of activation describing a transient activation followed by non-responsiveness. The stratgeies described above with anti-CD28 and anti-CTLA-4 antibodies are designed to overcome this significant problem.

Figure 9. In vivo activation kinetics of cytotoxic T cells in response to anti-CD3 antibody 2C11. Injections of 2C11 were administered on Day 0 and again on Day 10, 4 μg i.v., to male C57BL/6 mice. Injections were made across the 17 day period such that all animals were sacrificed and analyzed together. Splenocytes were harvested and tested the same day for cytotoxic activity using a standard ^{51}Cr-release assay, using FR positive F2-MTX^{r}A cells as targets, at the indicated E:T ratios. 2C11/folate conjugate (150 ng/ml) was used to redirect CTL activity toward F2-MTX^{r}A cells. Background release of ^{51}Cr in the absence of conjugate has been subtracted and in no case exceeded 10% specific release. Specific release was entirely competible by the addition of excess free folate. 2C11 caused an initial increase in cytotoxicity followed by a non-responsive period evident by minimal cytotoxicity following the second injection.

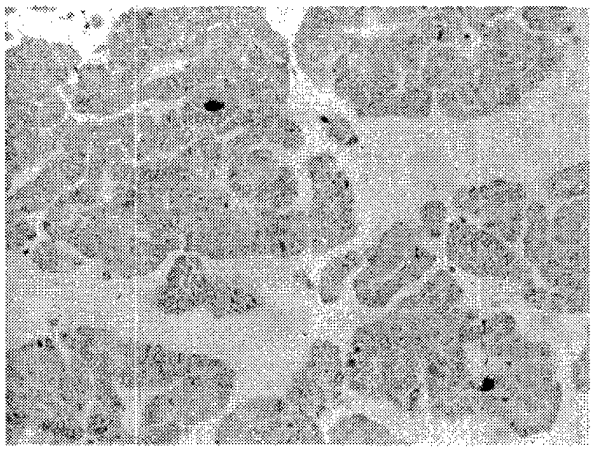


We have also shown that folate/anti-TCR antibodies are able to target T cells to brain tumors in this model (47). In these experiments, mice received a single i.p. injection (50 μg) of the T cell activating agent SEB one day prior to receiving antibody conjugates. Because the majority of the activated T cells express the Vβ8 region, the anti-Vβ8 antibody KJ16 was used for specific targeting. SV11 mice received i.v. injections of 10 μg of either KJ16 IgG, KJ16IgG/folate, KJ16scFv, or KJ16scFv/folate. To determine if T cells infiltrated the solid tumors, animals from each treatment group and the control were examined two days after antibody treatment by immunohistochemistry with an anti-CD3 antibody. In control mice that did not receive the SEB or antibodies, there were essentially no detectable CD3$^+$ T cells. In striking contrast, mice that received SEB followed by the scFv/folate conjugate had a major infiltration of CD3$^+$ T cells into the area of the tumor (Figure 10).

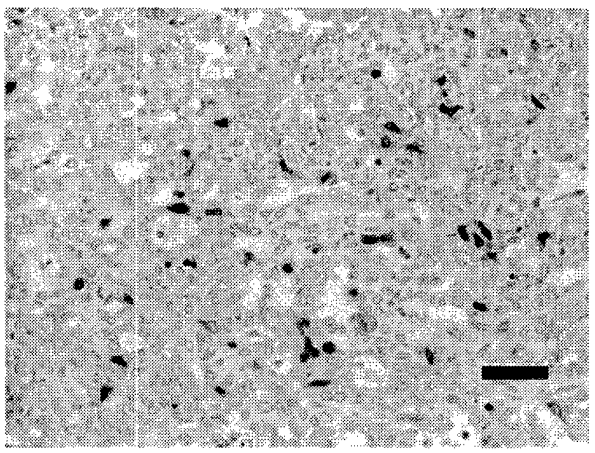
Figure 10. Infiltration of choroid plexus tumor by T cells following treatment with folate/anti-TCR bispecific antibody. **A.** Immunohistochemistry of T cell marker CD3 following treatment of SV11 mice with SEB (50 μg i.p.). Tumor cells are counterstained with methyl green. **B.** Immunohistochemistry of T cell marker CD3 following treatment of SV11 mice with SEB (50 μg i.p.) and scFv-KJ16 antibody (10 μg i.v.) reveals minimal T cell infiltration when folate is not conjugated to the antibody. **C.** Folate/scFv-KJ16. Immunohistochemistry of CD3 following treatment with SEB (50 μg i.p.) and Folate/scFv-KJ16 antibody (10 μg i.v.). Extensive infiltration of darkly staining T cells is observed. Scale bar is 50 microns.



SEB



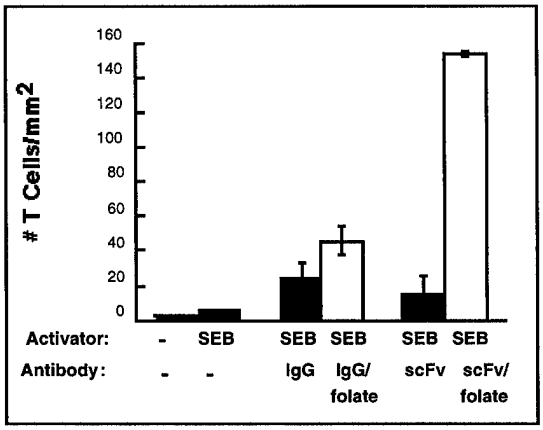
sc KJ16



sc KJ16 Folate Conjugate

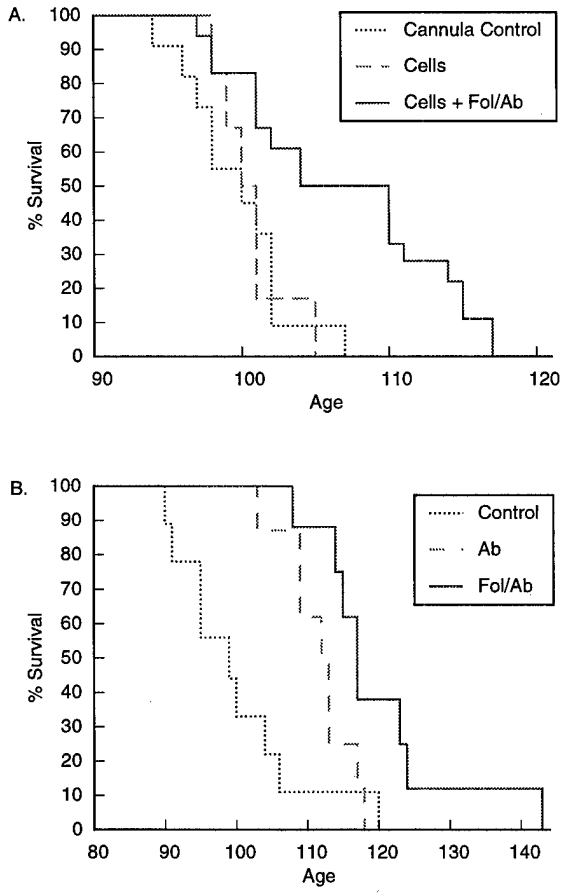
As quantified below, the most extensive T cell infiltration occurs only with the folate-labeled conjugates. Perhaps most impressive about the penetration following the scFv/conjugate was the uniform distribution of T cells well into the core of the solid tumor. Access to this region of tumors has been especially problematic, and it may be that the small size of the single-chain antibody and motility of T cells accounts for this property of bispecifc therapy. The scFv/folate conjugate was substantially more effective than the IgG/folate conjugate (Figure 11).

Figure 11. Quantification of T cell infiltration into SV11 brain tumors following treatment with KJ16 anti-TCR agents. Six sections per brain were analyzed using NIH Image for determination of tumor area, following by counting of stained cells.



We have conducted three experiments on the effect of antibody/Fol conjugates on survival in this model. In the first trial, we used the IgG form of 2C11, with or without folate conjugate, using an initial injection of 2C11 to activate T cells in both treatments. We observed a significant prolongation of survival with both treatments, but no additional benefit from the folate targeting. In a second trial, the folate/2C11 conjugate was administered intracerebroventricularly along with syngeneic splenocytes. Bispecific antibody conjugate treated animals lived significantly longer than animals treated with splenocytes alone (Figure 12 and ref (48)). Hypothesizing that smaller Fab or scFv bispecific constructs might better penetrate the brain and allow systemic treatment, in a third experiment we employed folate/scFv-KJ16 administered systemically after initial activation by SEB treatment. In this experiment, we observed a significant prolongation of survival with scFv-KJ16 and a further significant benefit from folate/scFv-KJ16 (Figure 12 and ref (47)). Thus we have observed a significant prolongation of survival with two quite different folate/antibody treatments. A major goal of our continuing efforts is to improve this partial efficacy by sustaining activation of T cells with tumor-dependent activation of T cells.

Figure 12. Prolonged survival following treatment of SV11 mice with scFv/folate conjugates. **A.** SV11 mice were treated by intracerebral injection through an indwelling cannula beginning at 85 days of age, with either saline, preactivated splenocytes without antibody, or preactivated splenocytes with Folate/anti-CD3 conjugate. Treatment with conjugate and cells significantly prolonged survival. **B.** At Day 84 mice in experimental groups received an injection of SEB (100 μg, i.p.), followed 18 hrs later with an injection of scFv KJ16 or scFv KJ16/Folate (25 μg, i.p.). Experimental groups received three additional injections of 25 μg of the same treatment over a 12 day period. scFv KJ16 treated mice lived significantly longer than control mice ($p < 0.05$), and scFv KJ16/Fol treated mice lived longer than control mice ($p < 0.01$) and significantly longer than scFv KJ16 treated mice ($p < 0.05$). Mean survival: control, 100 days; scFv KJ16, 112 days; scFv KJ16/Fol, 120 days.



CONCLUSIONS

Work Completed. Tasks shown below with an * have now been completed and significant progress was made on tasks indicated with an **. The past year focused on tasks that are underlined.

***Task 1,** Cloning, expression, and testing of erbB-2 single-chain antibody, months 1-12.

***Task 2,** Screening of tumor cell lines for susceptibility to CTL-mediated lysis using the anti-fluorescein bispecific antibody, months 1-12.

***Task 3,** Breeding of transgenic TCR/RAG$^{-/-}$ mice and testing of peripheral blood T cells for reactivity with 1B2 antibodies, months 1-20. It is anticipated that approximately 175 mice will be produced by the end of this period.

***Task 4,** Cloning, expression, and *in vitro* testing of bispecific single-chain scFv$_2$ antibody (1B2/erbB-2), months 13-24.

***Task 5,** Screening of tumor cell lines for increased susceptibility to CTL-mediated lysis when tumor cells are treated with: anti-erbB-2 antibodies, IFN-γ, TNF-α, estrogen, tamoxifen, months 13-30.

***Task 6,** Transplantation of various erbB-2^{+} tumor cell lines into TCR/RAG$^{-/-}$ mice and evaluation of tumor incidence, months 16-36. It is anticipated that approximately 15 mice per month will be used.

****Task 7,** Purification and *in vitro* testing of bispecific Fab$_2$ antibody (1B2/erbB-2), months 30-48.

***Task 8,** *In vivo* testing of bispecific antibodies in TCR/RAG$^{-/-}$ mice that have received human tumor transplants, months 30-48. It is anticipated that approximately 15 mice per month will be used. This task now includes a continuation of our efforts with the human KB tumor model and folate/antibody conjugates.

***Task 9.** Characterization of the KJ16/800E6 (anti-Vβ8/anti-erbB2) bispecific scFv$_2$, months 30-48.

****Task 10.** Expression of scFv$_2$ (1B2/800E6 and KJ16/800E6) in a yeast secretion system, months 30-48.

****Task 11.** Comparison of *in vivo* activation strategies in TCR/RAG$^{-/-}$ mice, months 30-48. This task includes our studies with anti-CD28 bispecific antibodies and soluble forms of the anti-CTLA-4 Fab fragments to sustain the activity of T cells.

Publications of the Principle Investigator During the Past Year (*Directly related to this project):

*Patrick, T. A., D. M. Kranz, T. A. Van Dyke, and E. J. Roy (1997) Folate Receptors as Potential Therapeutic Targets in Choroid Plexus Tumors of SV40 Transgenic Mice. *J. Neuro. Oncol.* 32: 111-123.

*Cho, B.K., T.A. Patrick, E.J. Roy, and D. M. Kranz (1997) Efficient CTL Lysis of Folate-Receptor-Positive Tumor Cells by Single-Chain Fv/Folate Conjugates. *Bioconj. Chem.* 8:338-346.

*Manning, T.M., L.A. Rund, M. Gruber, F. Fallarino, T. F. Gajewski, and D. M. Kranz (1997). Antigen Recognition and Allogeneic Tumor Rejection in $CD8^+$ TCR Transgenic/$RAG^{-/-}$ Mice. *J. Immunol.* 159:4665-4675.

Kieke, M. C., B.K. Cho, E.T. Boder, D.M. Kranz, and K.D. Wittrup (1997) Isolation of Anti-T Cell Receptor scFv Mutants by Yeast Surface Display. *Protein Engineering.* 10:1303-1310.

Manning, T. C., C. J. Schlueter, T. C. Brodnicki, E. A. Parke, J. A. Speir, K. C. Garcia, L. Teyton, I. A. Wilson, and D. M. Kranz (1998) Alanine Scanning Mutagenesis of an ab T Cell Receptor: Mapping the Energy of Antigen Recognition. *Immunity* 8: 413-426.

*Kranz, D. M., T. C. Manning, L. A. Rund, B. K. Cho, and E. J. Roy (1998) Targeting Tumor Cells with Bispecific Antibodies and T Cells. *J. Control. Release* 53:77-84.

*Roy, E. J., B. K. Cho, L. A. Rund, T. A. Patrick, and D. M. Kranz (1998) Targeting T Cells against Brain Tumors with a Bispecific Ligand-Antibody Conjugate. *Int. J. Cancer* 76: 761-766.

*Patrick, T. A., D. M. Kranz, J. F. Zachary, and E. J. Roy (1998) Intracerebral Bispecific Antibody Therapy Increases Survival of Animals Bearing Endogenously Arising Brain Tumors. *Int. J. Cancer.* In Press.

Cho, B.K., M. C. Kieke, E.T. Boder, K. D. Wittrup, and D. M. Kranz (1998) A Yeast Surface Display System for the Discovery of Ligands that Trigger Cell Activation. *J. Immunol. Meth.* In Press.

Manning, T.C. and D.M. Kranz (1998) Binding Energetics of T Cell Receptors: Correlation with Immunological Consequences. *Immunology Today.* Submitted

Manning, T.C., E.A. Parke, L. Teyton, and D. M. Karnz. (1998) Effects of CDR Mutations on the Affinity of an αβ T Cell Receptor: Measuring the Energy Associated with CD4/CD8 Repertoire Skewing. Submitted.

Kieke, M.C., E. T. Boder, L. Teyton, K. D. Wittrup, and D. M. Kranz (1998) Selection of Functionally Displayed T Cell Receptor Mutants from a Yeast Surface Library. Submitted.

*Rund, L. A., B. K. Cho, T. C. Manning, E. J. Roy, and D. M. Kranz (1998) A Transgenic Model to Test Bispecific Antibody Targeting of Endogenous T Cells Against Human Tumors. Submitted.

In addition, this project was selected for a platform presentation at the DOD *Era of Hope* meeting (Oct 31-Nov 4, 1997).

Future Work:

We have requested and have been granted a no-cost extension of this award through 9/30/99. There are sufficient funds remaining to partially support the following experiments:

- Preparation of a larger quantity of folate/anti-CD28 Fab conjugates for testing in the SV40 transgenic tumor model.

- Engineering of anti-CTLA-4 scFv for higher affinity, in order to increase the effectiveness of this agent in sustaining T cell activity. This project will involve display of the scFv-4F10 on the surface of yeast, including mutagenesis/library construction, and selection by flow sorting (as done with the anti-TCR scFv-KJ16, see Kieke *et al Protein Engineering*, 1997).

We are also pleased to report that the NIH has awarded an R01 grant (P.I. Edward Roy with myself as co-P.I.) which will begin in early 1999 to continue the studies on bispecific antibody targeting in the folate receptor models. It is our belief that these studies will prove to be of significance in the treatment of breast cancer, using similar bispecific antibody conjugates.

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Appendix

Papers that have been published or accepted for publication and were directly supported by this award are provided in the Appendix:

*Manning, T.M., L.A. Rund, M. Gruber, F. Fallarino, T. F. Gajewski, and D. M. Kranz (1997). Antigen Recognition and Allogeneic Tumor Rejection in $CD8^+$ TCR Transgenic/$RAG^{-/-}$ Mice. *J. Immunol.* 159:4665-4675.

*Kranz, D. M., T. C. Manning, L. A. Rund, B. K. Cho, and E. J. Roy (1998) Targeting Tumor Cells with Bispecific Antibodies and T Cells. *J. Control. Release* 53:77-84.

*Roy, E. J., B. K. Cho, L. A. Rund, T. A. Patrick, and D. M. Kranz (1998) Targeting T Cells against Brain Tumors with a Bispecific Ligand-Antibody Conjugate. *Int. J. Cancer* 76: 761-766.

*Patrick, T. A., D. M. Kranz, J. F. Zachary, and E. J. Roy (1998) Intracerebral Bispecific Antibody Therapy Increases Survival of Animals Bearing Endogenously Arising Brain Tumors. *Int. J. Cancer.* In Press.

Antigen Recognition and Allogeneic Tumor Rejection in CD8$^+$ TCR Transgenic/RAG$^{-/-}$ Mice[1]

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Three sources of help for the development of a CD8$^+$ CTL response have been described: the CD4$^+$ direct and indirect pathways and the CD8$^+$ direct pathway. In an effort to understand the minimal requirements for the development of a CTL response in vivo, we have bred mice transgenic for the 2C TCR onto a RAG$^{-/-}$ background. The 2C T cells in this animal are exclusively CD8$^+$ CTLs of a single specificity, and they exhibit altered thymic maturation compared with that of T cells from 2C TCR/RAG$^{+/+}$ mice. T cells from 2C TCR/RAG$^{-/-}$ mice can be activated to a high level in vivo by administration of a self-MHC-restricted antigenic peptide. The 2C TCR/RAG$^{-/-}$ mice are able to reject B7-negative allogeneic tumors bearing the appropriate peptide/MHC ligand p2C/L^d. These mice fail to reject syngeneic tumors, and their RAG$^{-/-}$ littermates lacking 2C T cells uniformly succumb to both allogeneic and syngeneic tumors. Moreover, blockade of B7 costimulatory molecules fails to prevent tumor rejection in the 2C TCR/RAG$^{-/-}$ mice, suggesting that allorejection is occurring independently of B7-mediated costimulation as well as in the absence of CD4$^+$ T cells. CTLs isolated from the site of the tumor during the period of rejection express the activation marker CD25 and are able to mediate ex vivo cytolysis of tumor cells bearing the appropriate Ag. These results suggest that in this TCR transgenic model with a very high precursor frequency, CTL development can occur in the absence of B7:CD28 costimulation and without CD4$^+$ help. *The Journal of Immunology,* **1997, 159: 4665–4675.**

Transplantation of tissue containing mismatched MHC Ags generally produces a vigorous alloreactive response. The strength of such a response has been attributed to a high frequency of T cells (~10%) capable of recognizing alloantigen. While pathogen-specific T cells generally recognize a foreign peptide in the context of self-MHC, alloreactive T cells usually recognize self peptides in the context of a foreign MHC molecule. Three possible sources of help have been described in the generation of an alloreactive CTL response: 1) the direct CD4$^+$ pathway, 2) the indirect CD4$^+$ pathway, and 3) a direct CD8$^+$ pathway (1). Where it has been examined in tumor models, an additional CD8$^+$ indirect pathway is predominant in CTL development (2, 3). The direct CD4$^+$ pathway involves Th cells that recognize antigenic peptide/MHC-II complexes on donor cells. The indirect CD4$^+$ pathway involves Th cells that recognize peptides derived from donor class I MHC presented by self-MHC class II molecules on self-APCs (4). The direct CD8$^+$ pathway involves CTL recognition of peptide/MHC class I complexes on donor cells and is considered to be highly dependent on the number of donor professional APCs in a graft (5). The CD8$^+$ indirect pathway consists of initial CTL cross-priming by host bone marrow-derived APCs and requires the uptake and presentation of tumor Ags on host MHC class I molecules (2, 3). The relative importance of the various sources of help for CTL activation is not fully understood, but the dominant pathway probably depends on the scenario encountered. Studies using CD4$^-$CD8$^-$ double knockout mice showed that such mice could generate alloreactive T cells and that these animals were able to reject allogeneic skin grafts (6). Despite this finding, a recent report using separate CD4$^-$ and CD8$^-$ knockout mice has stressed an absolute requirement for CD4$^+$ cells in the initiation of allograft rejection (7).

It is well established that two signals are required for the induction of an optimal T cell response. TCR ligation of antigenic peptide/MHC complexes provides signal 1, while costimulation, most importantly through CD28 ligation by B7 molecules, provides signal 2 (8; reviewed in Refs. 9 and 10). Ligation of the TCR in the presence of costimulation leads to proliferation, while TCR ligation in the absence of costimulation produces either no response or a state of hyporesponsiveness. Recently, it has been demonstrated that CD28:B7 ligation serves to prolong cell survival and prevent apoptotic death by up-regulation of the Bcl-xL molecule (11). The B7 family of costimulatory molecules has been shown to be important in both the development of a T cell response and, more specifically, in allograft rejection. This has led to many efforts to prevent T cell activation using strategies that allow signal 1, but block signal 2. For example, B7 blockade with CTLA4Ig (12) has shown protection in several animal models of graft rejection (13–15). However, it appears that costimulation either by IL-2 or through CD28:B7 interactions is not an absolute requirement for the development of all T cell responses (16–19).

The 2C TCR is one of the most well-characterized TCRs. The CTL clone 2C was initially isolated from a BALB.B mouse as an allospecific T cell that recognized L^d on the mastocytoma P815 (20). The binding affinity of the 2C TCR for its defined alloantigen p2C/L^d has been measured (21), and the crystal structure of the receptor is now known (22). In addition to its primary Ag, peptide p2C bound to L^d, the 2C TCR also binds the Ags SIYRYYGL/K^b (23), dEV-8/K^b (24), and many of the Vβ8-specific superantigens

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(25). T cell development has been characterized in 2C TCR transgenic mice with the use of the clonotypic Ab 1B2 (26–28). We have now crossed 2C TCR mice onto a RAG-1$^{-/-}$ background (29) to produce animals with a uniform population of resting CTL precursor cells. These TCR/RAG$^{-/-}$ mice (hereafter referred to as TCR/RAG mice) contain 95% CD8$^+$1B2$^+$ peripheral T cells and lack B cells and T cells of any other specificity. This strain provides an opportunity to examine the requirements for CTL activation in a defined system with well-characterized Ags.

To investigate the ability of 2C TCR/RAG mice to recognize alloantigen in vivo, the DBA/2-derived P815 tumor line (30), against which CTL clone 2C was originally selected, was used in various transplantation experiments. The P815 model of tumor rejection has also been studied extensively, and recent work has used various transfectants of this cell line to evaluate the effect of the costimulation by B7 family molecules that are ligands for CTLA-4 and CD28 (31–33). Possible immune recognition and elimination of P815 by 2C TCR/RAG mice was of interest because this system would lack the three well-characterized sources of help for the development of an alloreactive CTL response; there are no CD4$^+$ Th cells for either direct or indirect help, and because P815 does not express B7, there is not a clear source of signal 2 for direct CTL stimulation. Despite these deficiencies, we show that large allogeneic tumor burdens are indeed rejected in a CD8-dependent manner in the absence of B7-mediated costimulation or help from CD4$^+$ T cells.

Materials and Methods

Mice

2C TCR transgenic mice (27) were crossed with RAG-1$^{-/-}$ mice (29) obtained from The Jackson Laboratory (Bar Harbor, ME). F_1 mice were backcrossed to RAG$^{-/-}$ to yield TCR/RAG$^{-/-}$ mice. Mice were maintained in barrier cages at the University of Illinois animal care facility. BALB/c mice were obtained from Harlan Sprague Dawley (Indianapolis, IA). Transgenic mice used in experiments were between 6 and 12 wk of age. Additional experiments were performed using 2C TCR transgenic mice crossed onto a RAG-2$^{-/-}$ (34) background. These animals as well as DBA/2 mice were bred and housed at the University of Chicago animal care facility.

Cell lines and Abs

P815 is the mastocytoma-derived tumor line (30) against which T cell clone 2C was initially selected (20). EL-4 is a syngeneic (H-2^b) T lymphoma-derived cell line. T2-L^d is a human lymphoblastoid derived line deficient in peptide transport and transfected with the L^d gene (35). All cell lines were maintained in complete RPMI 1640 medium containing 5 mM HEPES, 10% FCS, 1.3 mM L-glutamine, 50 μM 2-ME, penicillin, and streptomycin. 1B2 is a mouse IgG1 mAb that is clonotypic for the 2C TCR. The Abs 53-6.7 (anti-CD8), RM4-5 (anti-CD4), 53.2.1 (anti-Thy-1.2), H1.2F3 (anti-CD69), 7D4 (anti-CD25), and 4F10 (anti-CTLA-4) were obtained from PharMingen (San Diego, CA). Ab 30-5-7, a mouse IgG2a anti-L^d mAb, is sensitive for peptide-bound L^d molecules (36). P198 is an immunogenic tum$^-$ variant of P815 that was isolated and grown as previously described (37).

Peptides

The peptides SIYRYYGL (23), dEV-8 (24), MCMV, p2C (38), and QL9 (39) were synthesized on an Applied Biosystems 430A instrument (Foster City, CA) using standard F-moc chemistry at the University of Illinois Biotechnology Center (Urbana, IL) and were analyzed for purity by mass spectrometry and for concentration by quantitative amino acid analysis. Peptides were purified by reverse phase HPLC on a C_{18} column eluting with a linear 5 to 60% acetonitrile gradient over 60 min in 0.1% trifluoroacetic acid.

Proliferation assays

Standard proliferation assays were performed in triplicate round-bottom wells of 96-well plates over a 72-h period in complete RPMI medium. [^{3}H]thymidine at 1 μCi/well was added during the last 18 h of the incubation period. Cells were collected and harvested on glass filters using a semiautomated cell harvester (Cambridge Technology, Inc., Watertown, MA). In some experiments, supernatant from rat splenocytes treated with Con A was added at a 10% volume to the assays. Con A supernatant was combined 2/1 with 20% α-methylmanoside to neutralize free Con A.

Cytotoxicity assays

Cytotoxicity was examined in standard ^{51}Cr release assays. Briefly, target cells were labeled with ^{51}Cr for 1 h at 37°C. Some 10^4 target cells/well were incubated with various numbers of effector cells for 4 h. Supernatants were harvested and assayed for specific lysis according to the formula: percent specific lysis = (counts per minute experimental − counts per minute spontaneous)/(counts per minute maximal − counts per minute spontaneous). In all cases spontaneous lysis was <25% of maximal lysis.

Tumorigenicity experiments

Tumor cells, generally 10^7 for i.p. experiments and 10^6 for s.c. experiments, were washed thoroughly in PBS and injected into either the peritoneal cavity or the left flank of mice. For s.c. tumors, growth was assessed weekly or biweekly using calipers. Mean tumor diameter was calculated as the average of two perpendicular measurements. Animals were killed when tumors reached diameters >25 mm, or if the mice became overtly ill or the tumor became ulcerated. Mice injected i.p. were examined daily. Survival was assessed as either the day of death or the point when moribund mice were killed. Observation of mice injected i.p. was continued through a 10-wk period.

Flow cytometry

Flow cytometry was performed at the University of Illinois Flow Cytometry Facility using a Coulter Epics XL instrument. Thymi or spleens were removed from mice and passed through wire mesh, and RBC were lysed. Generally, ~10^5 cells/tube were incubated for 30 min with the appropriately labeled Ab in a volume of 50 μl PBS/1.0% BSA on ice. Cells were washed and resuspended in 400 μl before analysis.

Treatment of mice with murine CTLA4IgG3 fusion protein (mCTLA4γ3)[3]

Purified mCTLA4γ3 (32) and control IgG3 were adjusted to 100 μg/ml in PBS. Mice received 1 ml of either of these reagents on days −1, 0, 1, 2, 4, 7, 11, 14, 17, 21, and 24. Tumor cells were implanted several hours following the second injection on day 0.

Results

Characterization of TCR/RAG mice

The progeny of TCR/RAG$^{-/-}$ F_1 × RAG$^{-/-}$ mice were examined for serum IgM levels and 1B2$^+$ PBL. Mice that exhibited no detectable IgM by ELISA or 1B2$^+$ cells by flow cytometry served as founders for a colony of 2C TCR/RAG mice (data not shown). Two-color flow cytometry analysis using anti-Thy 1.2, and the clonotypic Ab 1B2 revealed that essentially 100% (99.4 ± 0.7%) of the T cells from the spleens of these mice were 2C T cells (Fig. 1). This showed not only that the mice express the transgenic TCR, but it confirmed the RAG$^{-/-}$ background of these mice, as no endogenously rearranged TCR genes (i.e., Thy 1.2$^+$/1B2$^-$ cells) were observed. This contrasts with 2C TCR transgenic mice (hereafter referred to as TCR mice), in which only 88 ± 5% of the splenic T cells were 1B2$^+$ ($p < 0.001$).

T cells from spleens and thymi of mice at different ages were examined using 1B2 and anti-CD4 and anti-CD8 Abs. Three-color flow cytometric analysis of 2C T cells from the thymus revealed that TCR/RAG mice had more CD4$^+$/CD8$^+$ cells and fewer CD4$^-$/CD8$^-$ than did the TCR mice (Fig. 2*A*). Within the TCR/RAG thymus, 35 ± 12% of the 2C thymocytes were CD4$^+$CD8$^+$, while in the TCR thymus, only 19 ± 7% of the 2C thymocytes were CD4$^+$CD8$^+$ ($p < 0.01$). Conversely, the number of CD4$^-$CD8$^-$ cells was reduced (24 ± 9%) in TCR/RAG mice compared with that in TCR mice (41 ± 9%; $p < 0.005$). Thus,

[3] Abbreviations used in this paper: mCTLA4γ3, murine CTLA4IgG3 fusion protein; ^{low}, low level; PEC, peritoneal exudative cell.

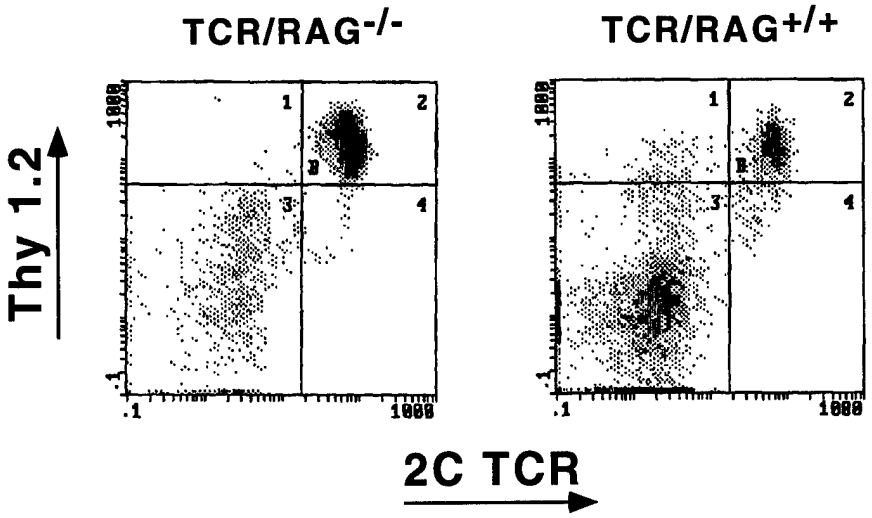

FIGURE 1. Characterization of T cell populations in TCR/RAG and TCR mice. Flow cytometry was performed as described in *Materials and Methods* using the mAbs 53.2.1 (anti-Thy 1.2) and 1B2 (2C TCR clonotypic).

TCR/RAG mice had about twice as many double-positive thymocytes and about half as many double-negative thymocytes as TCR mice. The significant number of $CD4^-CD8^-$ 2C T cells present in the spleens of TCR mice was also absent in the spleens of TCR/RAG mice (Fig. 2*B*). Within the spleens of TCR/RAG mice, 95 ± 2% of 2C T cells were $CD8^+$, while in spleens of TCR mice, only 65 ± 10% of 2C T cells were $CD8^+$, with the majority of the remainder being $CD4^-CD8^{low}$ or $CD4^-/CD8^-$ ($p < 0.0001$). Thus, in the TCR/RAG mice, ~95% of the entire T cell repertoire consists of $CD8^+$ CTL precursors, while in the TCR mice, only about 55% (45–70%) of all T cells are 2C $CD8^+$ CTL precursors. These results indicate that the prevention of endogenous TCR gene rearrangement during thymic development in the 2C TCR/RAG mouse leads almost exclusively to a $CD8^+$ CTL phenotype.

Ability of TCR/RAG to reject allogeneic tumors

Since 2C TCR/RAG mice lack B cells or Th cells, they provide a unique opportunity to examine the requirements for the development of a CTL response. The allogeneic tumor line P815 lacks the B7 family of costimulatory molecules and thus provides a source of signal 1 (p2C/L^d-TCR ligation) in the absence of signal 2 (B7-CD28 ligation). To investigate whether TCR/RAG mice could reject an allogeneic tumor, P815 cells were injected either s.c. or i.p.

In the i.p. model, control $RAG^{-/-}$ animals ($n = 5$) injected with 10^7 cells failed to reject the allogeneic tumors and died or became moribund and were killed within 3 wk (Fig. 3*A*). TCR/RAG mice ($n = 5$) injected with 10^7 cells showed no signs of illness and survived the 10-wk period, with one exception (discussed later). Survival of TCR/RAG mice was significantly prolonged compared with that of $RAG^{-/-}$ mice ($p < 0.005$). To examine the strength of the response, mice were injected with a 30-fold greater tumor load (3×10^8 cells). Survival of these high tumor load animals ($n = 3$) was also prolonged compared with that of $RAG^{-/-}$ mice ($p < 0.05$), but only one of the animals survived through 10 wk. without event. TCR/RAG mice injected with 10^7 cells appeared to survive longer than those receiving 3×10^8 cells, although this result was not statistically significant ($p = 0.10$). In the s.c. tumor model, $RAG^{-/-}$ mice injected with 10^6 P815 cells uniformly developed tumors that grew to a large size within 3 wk, while the TCR/RAG animals remained free of tumors and exhibited no illness throughout a 6-wk period (Fig. 3*B*). Tumor recurrence in these animals had not been observed through day 60 ($n = 3$). The fact that tumors were rejected by TCR/RAG mice but not by $RAG^{-/-}$ mice indicates that the transgenic $CD8^+$ CTL is necessary for the rejection.

To confirm the specificity of tumor rejection, the response of 2C TCR/RAG mice to s.c. injection of syngeneic EL4 tumor cells ($H\text{-}2^b$) was examined. EL4 tumors grew rapidly, while P815 tumors were uniformly rejected (Fig. 4). Coinjection of equivalent numbers of EL4 cells together with P815 cells delayed the growth of EL4 tumors by about 1 wk ($p < 0.0005$), but once established, growth rates for the EL4/P815 mix were similar to those for EL4 alone. Thus, while some bystander activity or peptide/MHC-independent killing may be responsible for slowing the initial growth of EL4, 2C CTLs are only able to cause the rejection of tumors bearing Ag of the correct specificity (i.e., p2C/L^d).

Tumor recrudescence is not attributable to tumor variant escape

Two of the high tumor dose i.p. animals and one of the lower tumor dose i.p. animals exhibited tumor reoccurrence during the course of the 10-wk observation period. Analysis of one of these animals revealed a large ascites tumor yielding over 4×10^8 cells. Microscopic examination of a Wright's stained smear of this tumor demonstrated two distinct populations of cells: a smaller cell type appearing identical with P815 and a larger mononuclear granulocyte (data not shown). After culture for several days, a single cell population became dominant, which appeared identical with cultured P815 by microscopic examination. Flow cytometric analysis of the peritoneal lavage demonstrated that the recovered cells consisted of both L^{d+} P815 tumor cells and a large number of activated peritoneal macrophages that were Thy 1.2^- and $CD25^+$ (Fig. 5*A*). Staining with the anti-L^d Ab 30-5-7 demonstrated that the levels of L^d were identical for the recovered tumor cells and cultured P815 (data not shown).

A CTL killing assay confirmed that both the recovered P815 tumor and cultured P815 were identical in their susceptibility to lysis by activated 2C CTLs (Fig. 5*B*). The responsiveness of the 2C T cells from these animals was not investigated, but it is possible that TCR/RAG mice have defects associated with memory responses, as has recently been observed in a TCR transgenic model of viral immunity (40). Thus, it appears that while tumor control is generally achieved and maintained, in at least some of the animals there is a failure to completely eradicate tumor cells, and late regrowth of tumor can occur. Moreover, this tumor recrudescence is not attributable to down-regulation of L^d by the tumor cells or resistance to CTL-mediated killing.

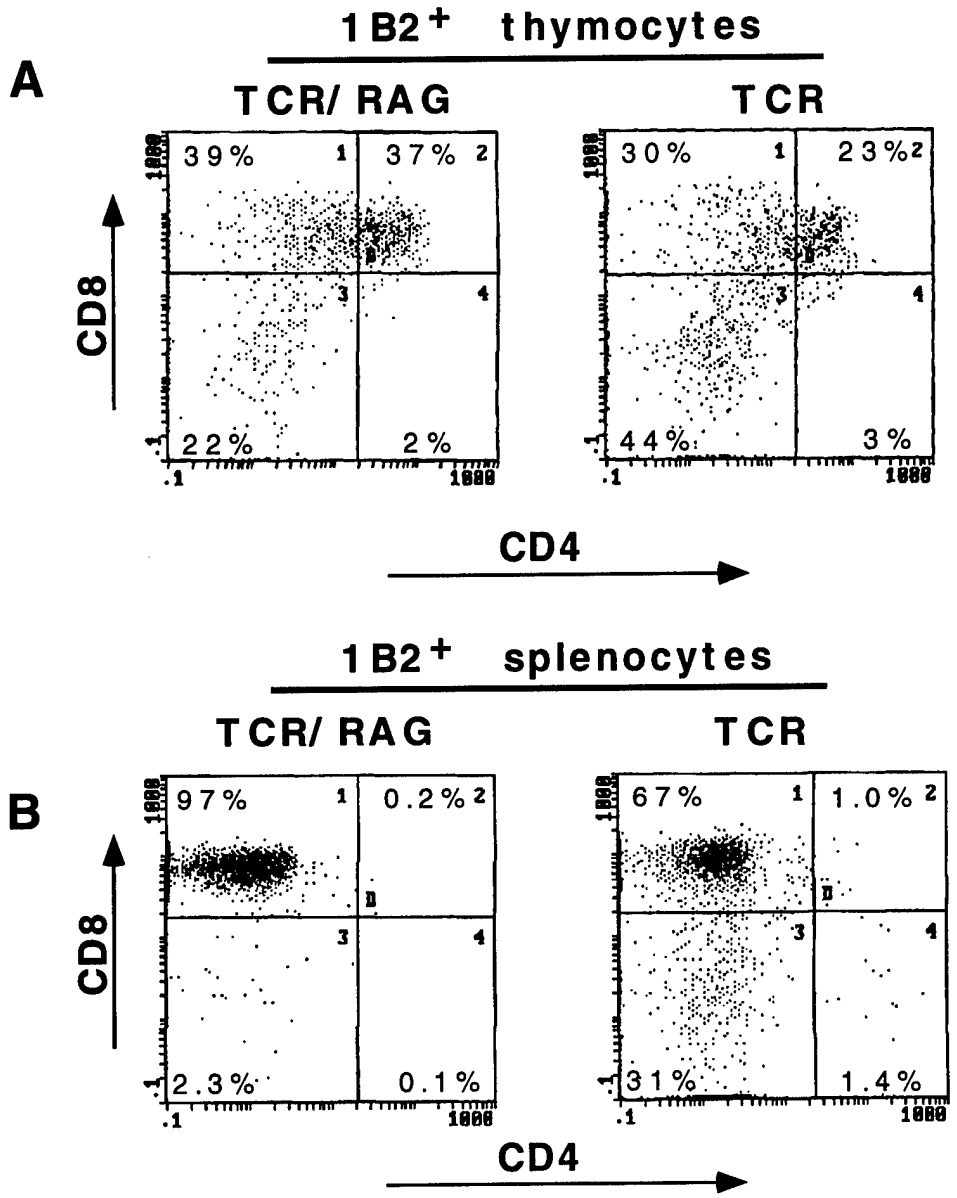

FIGURE 2. Comparison of 2C T cell populations from thymus and spleen of TCR/RAG and TCR mice. Flow cytometry was performed as described in *Materials and Methods* using the mAbs 1B2 (2C TCR clonotypic), 53-6.7 (anti-CD8), and RM4-5 (anti-CD4). *A*, The 1B2$^+$ population of thymocytes was analyzed for CD4 and CD8 expression by three-color staining. *B*, The 1B2$^+$ population of splenocytes was analyzed for CD4 and CD8 expression by three-color staining. Representative results for a single animal of each type are shown. Percentages given in the text reflect the average of six animals of each type.

Rejection of allogeneic tumors is B7 independent

While P815 is a B7-negative tumor, it remained possible that host B7 molecules might be playing a role in costimulating 2C CTL development during a P815 tumor challenge. Such costimulation might be delivered either by host APCs acting in a bystander or *trans* manner or, alternatively, through the processing of an unknown tumor Ag through the CD8$^+$ indirect pathway. To assess whether a role for host B7 molecules exists in the rejection of B7-negative P815, mice were treated with mCTLA4γ3. The efficacy of the mCTLA4γ3 used in the experiments was confirmed by its ability to prevent rejection of the immunogenic P198 tumor in immunocompetent DBA/2 mice (Fig. 6*A*). The ability of B7 blockade to prevent rejection in this model has been taken as evidence for costimulation by host B7 in the rejection of B7-negative tumors (32). Treatment of 2C TCR/RAG mice with an identical course of mCTLA4γ3 or control IgG3 had no effect on the ability of these mice to reject s.c. P815 tumors (Fig. 6, *B* and *C*). RAG$^{-/-}$ mice injected with P815 uniformly grow large s.c. tumors within 3 wk (Fig. 6*B*), while both the mCTLA4γ3 and control treated groups are indistinguishable in their ability to reject these same tumors. Further, the same results were observed independent of the background of the mice (i.e., either RAG-1$^{-/-}$ (Fig. 6*B*) or RAG-2$^{-/-}$ (Fig. 6*C*)). These results illustrate that the observed tumor rejection is independent of both CD4-derived help and B7-mediated costimulation.

Characterization of the effector cell population during tumor rejection

While it was clear that 2C CTLs were necessary for the tumor rejection observed in the TCR/RAG mice, it was not clear how

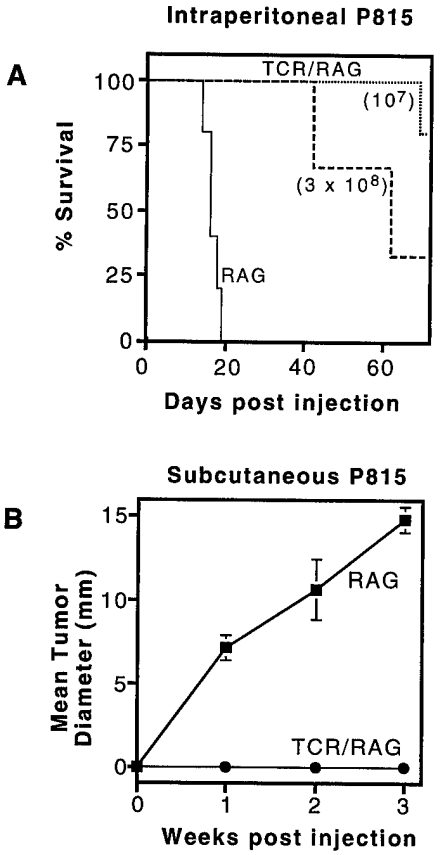

FIGURE 3. Tumor allograft rejection in TCR/RAG mice. *A*, TCR/RAG mice were injected i.p. with either 10^7 P815 cells (n = 5 mice) or 3 × 10^8 P815 cells (n = 3 mice). RAG mice received injections of 10^7 P815 cells (n = 5 mice). Animals were monitored as described in *Materials and Methods* for a period of 10 wk. *B*, TCR/RAG or RAG mice (n = 3) were injected s.c. in the left flank with 10^6 P815 cells in 100 μl and monitored weekly for tumor growth. The mean tumor diameter represents the average of two perpendicular measurements.

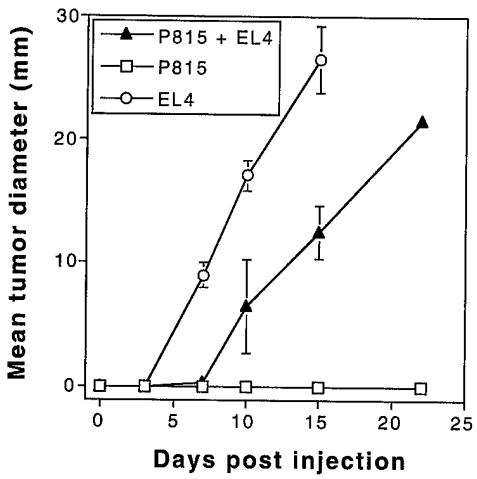

FIGURE 4. Tumoricidal activity in TCR/RAG mice is L^d restricted. TCR/RAG mice were injected s.c. in the left flank with 10^6 EL4 (H-2^b) or P815 (H-2^d) in 100 μl of PBS. Mice receiving coinjection of both cell types received 10^6 of each in a single injection. Mice were monitored as described in *Materials and Methods*. The absence of error bars indicates a SD smaller than the size of the symbol.

these CTLs were accomplishing this task in the absence of any of the established sources of help for a CTL response. To explore the mechanism of tumor rejection, splenocytes and peritoneal exudative cells (PECs) from animals at various times following i.p. P815 injection were examined. Flow cytometric analysis of splenocytes exhibited only very low or background levels of the activation markers CD69, CD25, and CTLA-4 throughout the course of tumor rejection (data not shown). Further, only a low level (12%) of ex vivo cytolysis of P815 target cells was observed by splenocytes on day 4 (Fig. 7). This compares to cytolysis levels of typically >50% for cultured 2C effectors.

To assess whether 2C T cells were activated at the site of the tumor during the period of rejection, PECs were isolated from mice 4 days after i.p. injection of P815 tumor cells. When examined by flow cytometry, PECs showed blastic changes and stained positively for the T cell activation marker CD25, as well as for 2C TCR and CD8 (Fig. 8). In addition to $CD25^+$ blastic 2C T cells (Fig. 8, *B* and *C*), the peritoneal exudate consisted of a subset of smaller $CD25^-$, $CD8^+$ 2C T cells (Fig. 8*A*) as well as a more granular non-T cell population (Fig. 8*D*). The ability of PECs to lyse L^d-bearing target cells (T2-L^d) that were loaded with the peptide QL9 was examined. Ex vivo cytolytic activity of PECs varied from a low of 3% to a high of 40%, averaging 23% in the presence of the peptide QL9 (Table I). A variable amount of peptide-independent lytic activity averaging about one-third of the total lytic activity was observed in the unfractionated PECs. The peptide-dependent lytic activity (about two-thirds of the total activity) was inhibited to the peptide-independent level by addition of the anti-clonotypic Ab 1B2. This result suggests that the remaining peptide-independent lysis is not attributable to killing mediated through the 2C TCR (Table I). The response of TCR/RAG mice to an i.p. injection of BALB/c splenocytes (a B7-positive source of the p2C/L^d Ag) was compared with that to an i.p. injection of P815. The results indicated no significant difference in either CD25 levels or lytic activity of 2C T cells isolated from the peritoneum (data not shown).

In vitro recognition of alloantigen

It has previously been reported that purified $CD8^+$ 2C CTLs do not proliferate when cultured with P815 tumor cells unless exogenous IL-2 is added (41). We sought to confirm this result and determine whether there were any differences that might explain the rejection of P815 tumors by the TCR/RAG mouse. A comparison of the proliferative response to alloantigen in the form of either P815 tumor cells or BALB/c splenocytes (both L^d positive) revealed a marked disparity depending on the nature of the APC (Fig. 9). Proliferation increased as the density of BALB/c APC increased, while proliferation decreased at higher P815 stimulator densities. In the case of both BALB/c and P815, addition of exogenous cytokines increased the proliferative responses. Depending upon the number of stimulator cells used in a single point assay, the ratio of proliferative responses to BALB/c vs P815 ranged from as high as 500:1 to as low as 2:1. When exogenous cytokines were present, this ratio varied from a high of 12:1 to a low of 1:1.5. Thus, it is evident that TCR/RAG splenocytes are indeed capable of proliferation in response to P815 tumor cells (21,000 cpm maximally), but at high densities of stimulators the response to BALB/c far exceeds the response to P815.

In vivo activation by self-MHC-restricted Ag

To determine whether the 2C CTLs were capable of full activation in vivo, soluble antigenic peptides were injected i.p. on 2 consecutive days, and splenocytes were examined for activation on the third day. The peptides included MCMV, a K^b-restricted peptide that is not recognized by the 2C TCR; p2C and dEV-8, peptides that in complex with K^b are recognized with low affinity ($K_a \cong$

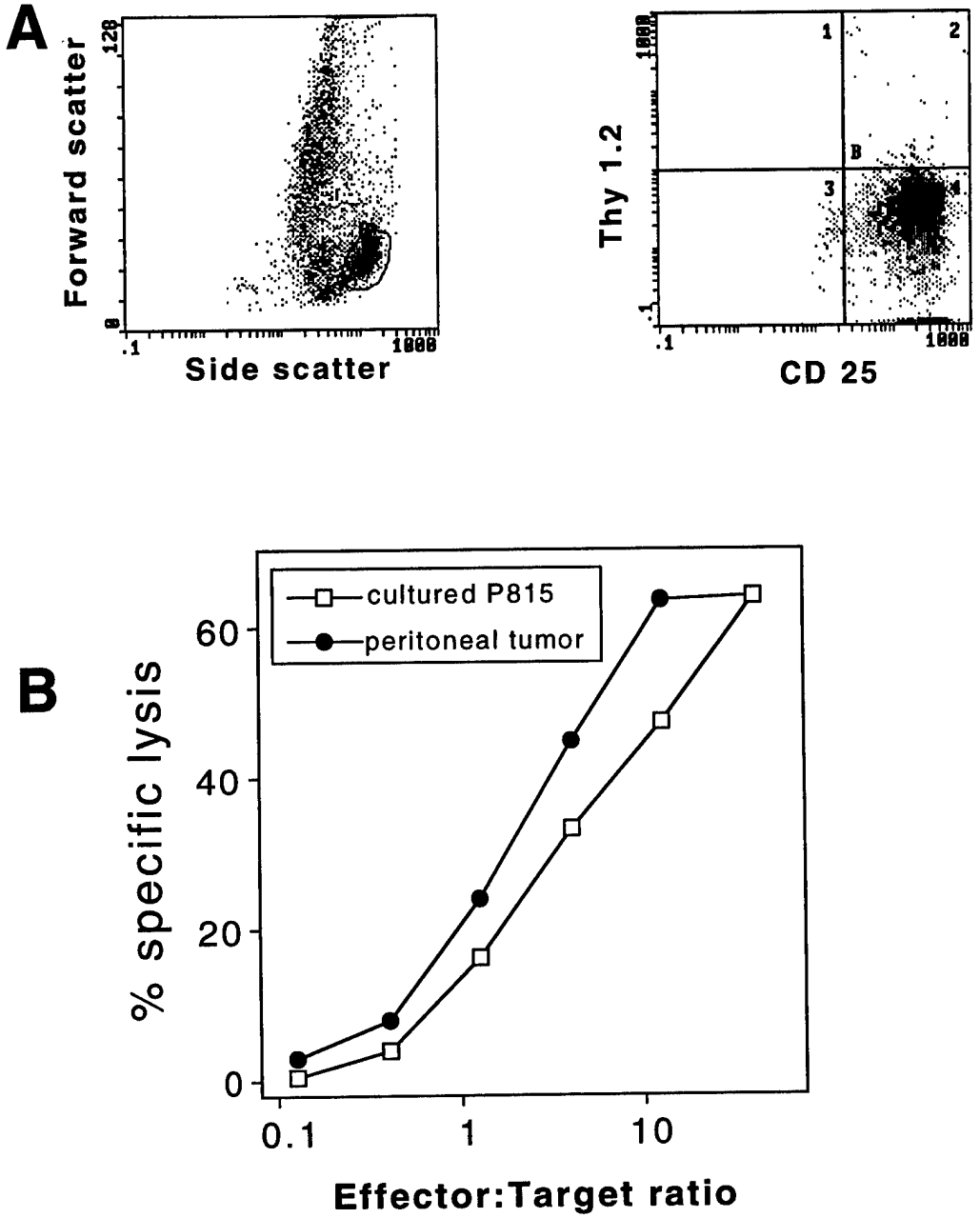

FIGURE 5. Characterization of peritoneal cell population in a TCR/RAG mouse with late tumor reoccurrence. Cells (4.5×10^8) were harvested by peritoneal lavage of an ascites tumor from a moribund mouse injected 69 days earlier with 10^7 P815 cells. *A*, Flow cytometric analysis of the cell populations isolated from the peritoneum of the same animal. The population of macrophages was gated and analyzed for expression of Thy 1.2 and CD25. *B*, Ascites tumor cells that had been in culture for 5 days and cultured P815 were assayed in a standard 4-h ^{51}Cr release killing assay for susceptibility to lysis by in vitro activated 2C CTLs.

3×10^3 and 2×10^5 M^{-1}, respectively) by CTL 2C (21, 22); and SIYRYYGL, a synthetic peptide that was selected from a peptide library based on its ability to sensitize K^b-bearing cells to lysis by 2C (23). Only the SIYRYYGL peptide was able to vigorously activate T cells, as measured by expression of the activation marker CD69 24 h after injection (Fig. 10*A*). Essentially 100% of the $1B2^+$ T cells in the spleen become $CD69^+$ following exposure to this peptide.

To confirm that these activated cells were also capable of cytolysis, the ability of splenocytes from peptide-injected animals to lyse P815 target cells was examined. Again, only the SIYRYYGL peptide elicited splenocytes able to lyse L^d-bearing target cells ex vivo (Fig. 10*B*). Interestingly, injection of the peptide dEV-8, a putative positively selecting peptide for CTL 2C, also failed to produce detectable ex vivo cytolytic activity (Fig. 10*B*) or activation marker up-regulation (data not shown). Thus, 2C CTLs in the TCR/RAG could be fully activated after in vivo Ag challenge, but presumably only the higher affinity peptide/K^b or TCR/peptide/K^b interactions could achieve this activation. It is thought that the 2C TCR recognizes both p2C/L^d and SIYRYYGL/K^b with similar high affinity (23). These results confirm that the 2C CTL precursor cells in the TCR/RAG mice are indeed capable of full and complete activation in response to the appropriate peptide/MHC Ag. It suggests that the low levels of activation observed in the spleen after injection of P815 may be attributable to a lower load of Ag and localization of the Ag to the peritoneum.

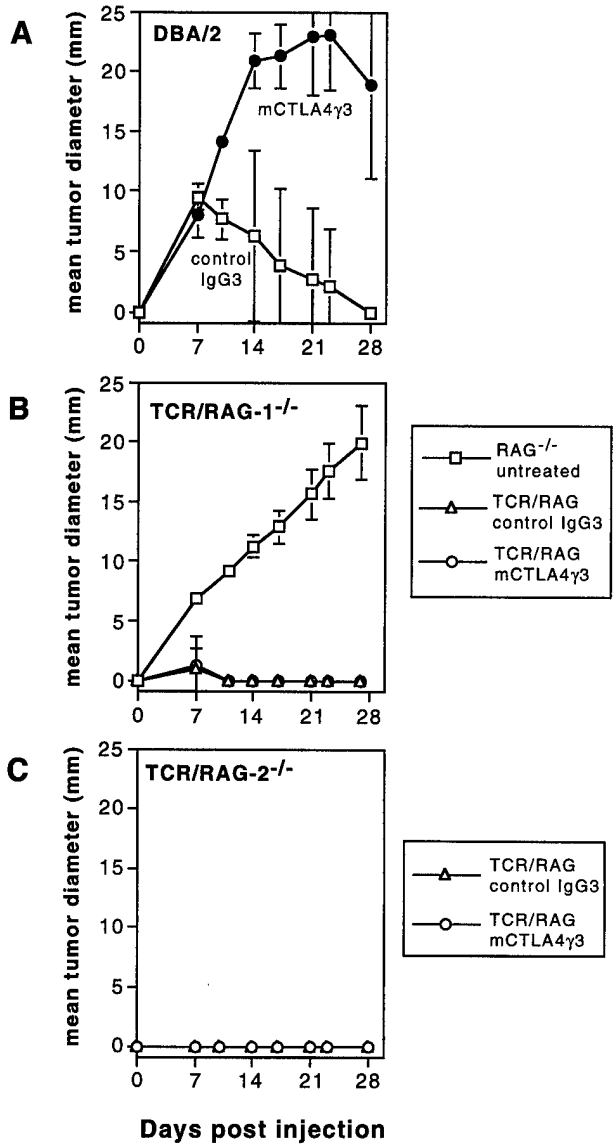

FIGURE 6. Role of B7 molecules in rejection of tumors. Mice were treated with a series of i.p. injections of either mCTLA4γ3 or control IgG3 beginning on day −1 as described in *Materials and Methods*. Tumor cells were injected on day 0 several hours after the second dose of mCTLA4γ3. *A*, DBA/2 mice were injected with 10^6 P198 tumor cells in 100 μl of PBS in the left flank and monitored biweekly for tumor growth. *B* and *C*, 2C TCR/RAG mice on either a RAG-$1^{-/-}$ background (*B*) or a RAG-$2^{-/-}$ background (*C*) were injected with 10^6 P815 tumor cells in 100 μl of PBS in the left flank and monitored biweekly. The mean tumor diameter represents the average of two perpendicular measurements. The absence of error bars indicates an SD smaller than the size of the symbol.

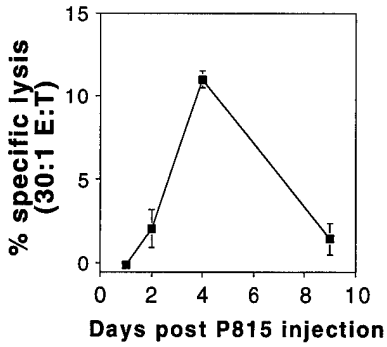

FIGURE 7. Kinetics of T cell activation in TCR/RAG mice during allorejection. Ex vivo cytolysis of splenocytes isolated on various days following tumor injection. Splenocytes were assayed against ^{51}Cr-loaded P815 target cells at various E:T ratios. The lysis at a 30:1 E:T ratio is shown. Each point represents a single mouse. Error bars represent the SD of triplicate wells within the same assay. Fully activated 2C CTLs typically show >50% specific lysis at 30:1 E:T against P815 (data not shown).

Discussion

The critical importance of costimulation in T cell-mediated responses has been repeatedly demonstrated within the last several years in numerous systems. Nevertheless, several situations have now been described where the most well-characterized costimulation signal, CD28:B7, is not an absolute requirement (16–19). We now present a system where allogeneic tumors are rejected in a B7-independent manner by a mouse that possesses only $CD8^+$ CTLs of a single specificity and that completely lacks B cells or Th cells.

Thymic development of 2C T cells appears to proceed along a slightly different kinetic course in TCR/RAG mice compared with TCR mice. Fewer thymocytes mature into $CD4^-CD8^-$ peripheral T cells in the TCR/RAG (5%) than in the TCR (45%). In contrast, there are proportionally twice as many $1B2^+CD4^+CD8^+$ thymocytes in the TCR/RAG thymus as in the TCR thymus. Mice transgenic for a TCR that was specific for MHC-I demonstrated increases in thymic emigrant numbers, but the increase was accompanied by a reduced size of the $CD4^+CD8^+$ thymocyte pool (42). This reduced double-positive pool is not observed when the transgenic 2C TCR is expressed on a RAG background. The explanation for why there are more mature $CD4^-CD8^-$ 2C T cells in the periphery of TCR mice than in TCR/RAG mice is not clear. The $CD8^+$ and $CD8^-$ subsets of 2C cells in the TCR animal are distinct and nonoverlapping and have been purified by others (43). In the TCR mouse, transient expression of endogenous TCR chains during thymic development might reduce the number of transgenic 2C receptors. A reduction in 2C TCR density may lead to altered signaling and a resultant loss of both CD4 and CD8 expression from 2C cells. Variations in the number of TCR per cell required for signaling various T cell functions has recently been described (25, 44).

The fact that 2C TCR/RAG mice are able to reject even large tumor burdens while their corresponding RAG$^{-/-}$ littermates succumb to the tumors indicates that the cell type responsible for this allorejection is the $CD8^+$ CTL bearing the 2C TCR. Our s.c. dose of 10^6 P815 cells is 20-fold greater than the minimal tumorigenic dose used in many syngeneic animal models (31), while our i.p. tumor dose is 10-fold greater still. Because this animal lacks $CD4^+$ T cells or, in fact, T cells that might recognize any other Ag, the CTL response must be independent of CD4 help. What, then, is the source of help necessary for the rejection of these tumors? There are several possibilities. First, it may be that the rejection is made possible by the sheer number of CTL precursors and the high intrinsic affinity (2×10^6 M^{-1}) of the 2C TCR/p2C/L^d interaction, which could abrogate the requirement for costimulation, as has been proposed previously (43). That is, since p2C is immunodominant for L^d, and P815 expresses high levels of L^d, the high epitope density and high TCR affinity may act to overcome the requirement for costimulation. A sufficient number of CTLs may secrete their own cytokines and become activated locally to a level that is capable of initiating the lytic response and controlling the tumor.

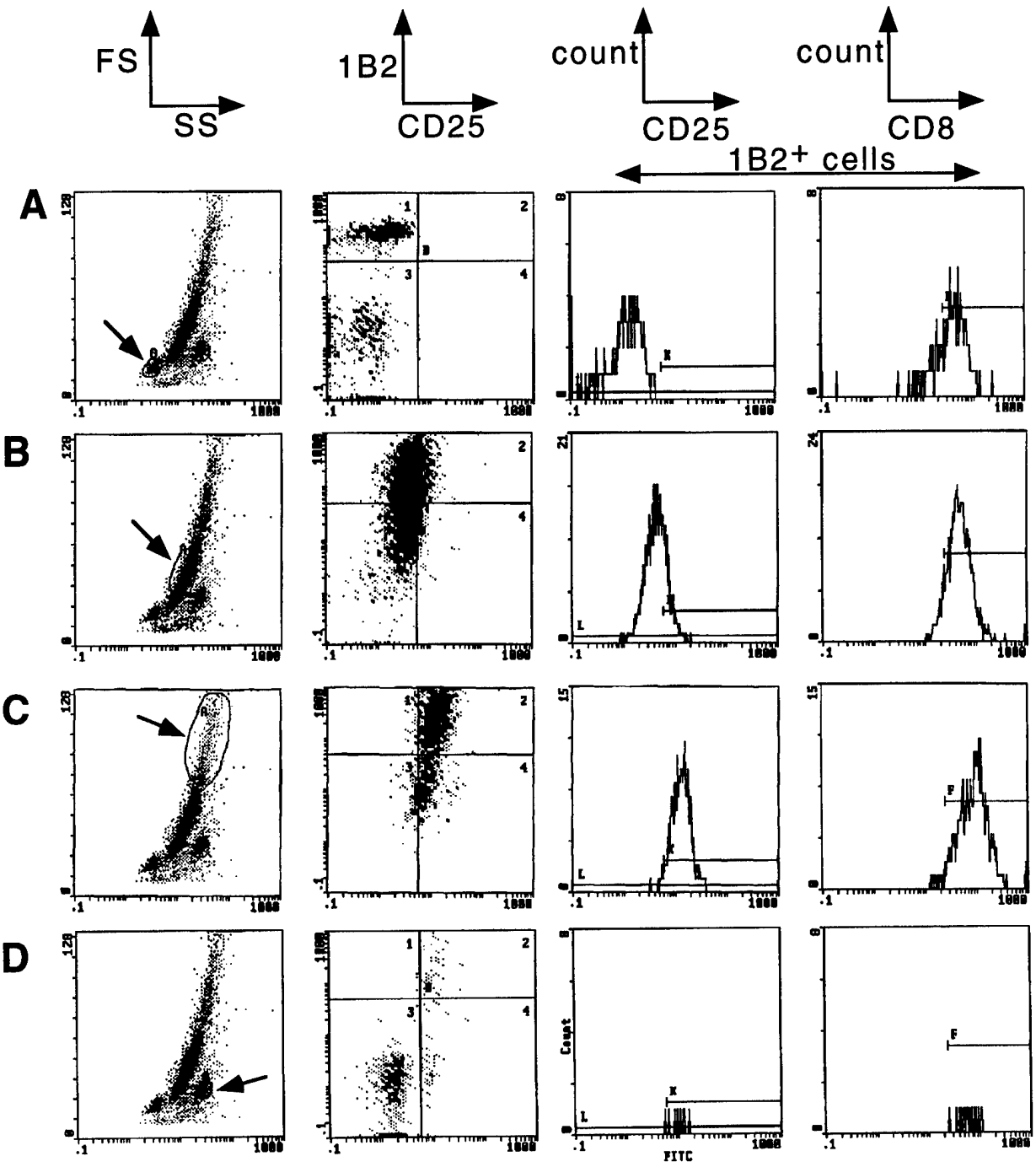

FIGURE 8. PEC populations at the time of tumor rejection. PECs were isolated by peritoneal lavage from mice injected with P815 tumor cells 4 days earlier. Distinct populations were gated upon (gate indicated by arrow) and analyzed for various T cell markers by flow cytometry. *A*, Smaller CD25$^-$ 2C CTL precursors. *B* and *C*, Larger, blastic CD25$^+$ 2C CTLs. *D*, Population of more granular, non-T cells. Results were representative of several mice analyzed.

A second explanation may involve an interplay between T cells and the innate immune system. It has been shown that macrophages and other cell types, such as NK cells, can participate in cytokine cascades that direct a T cell response toward either Th1 or Th2. Moreover, a requirement for NK cells in the induction of alloreactive human CTLs has been demonstrated (45). Such an interplay with the innate immune system operating in the local environment of the peritoneal cavity or an s.c. site might provide the necessary help needed for the development of CTL precursors into lytic CTLs. In Figure 9, the low level of proliferation in response to P815 is shifted nearly fourfold by the addition of exogenous cytokines. Thus, it is possible that other cell types in the local environment might be providing the requisite cytokines. For example, the IL-1β/IL-12/IFN-γ cytokine cascade involves macrophages and NK cells (46). IL-12 secreted by macrophages or dendritic cells is known to be a potent inducer of Th1/Tc1 responses and is probably acting along with Ag to help promote CTL development.

A third possibility is that other costimulatory pathways could be replacing B7:CD28 in producing CTLs capable of tumor cell lysis. P815 is known to express intercellular adhesion molecule-1, which binds the LFA-1 Ag on T cells and can provide costimulatory

Table I. *Lysis of ^{51}Cr-labeled T2-L^d target cells by TCR/RAG PECs*[a]

Mouse	% Lysis by PECs at 30:1 E:T (+QL9)	(No Peptide)	% Peptide Dependent	% 1B2 Inhibition (+QL9)
1	33	20	40	n.d.
2	20	14	30	n.d.
3	40	4.6	89	91
4	33	0.8	97	83
5	2.8	0.4	86	89
6	8.3	3.0	64	54
Average	23	7.1	68	

[a] Effector cells were obtained by peritoneal lavage 4 days following i.p. injection of 10^7 P815 cells. Assays were performed as described in *Materials and Methods*. Maximal 1B2 inhibition was assessed at a 1B2 concentration of 3–10 μg/ml. n.d. indicates that 1B2 inhibition was not examined in the indicated mouse.

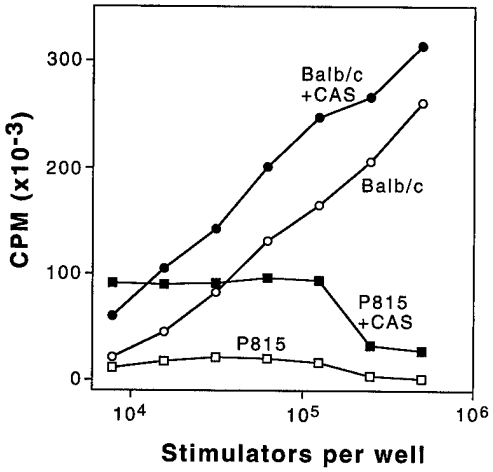

FIGURE 9. In vitro proliferation of 2C CTL precursors in response to alloantigen. Responder TCR/RAG splenocytes were cultured at 5 × 10^4 cells/well with various numbers of allogeneic stimulator cells. Exogenous cytokine-rich supernatant from Con A-treated rat spleens (CAS) was added where indicated at a 10% concentration. Proliferation of stimulators or responders cultured alone yielded background levels <1500 cpm. The maximum proliferation in the presence of P815 without CAS was 21,000 cpm.

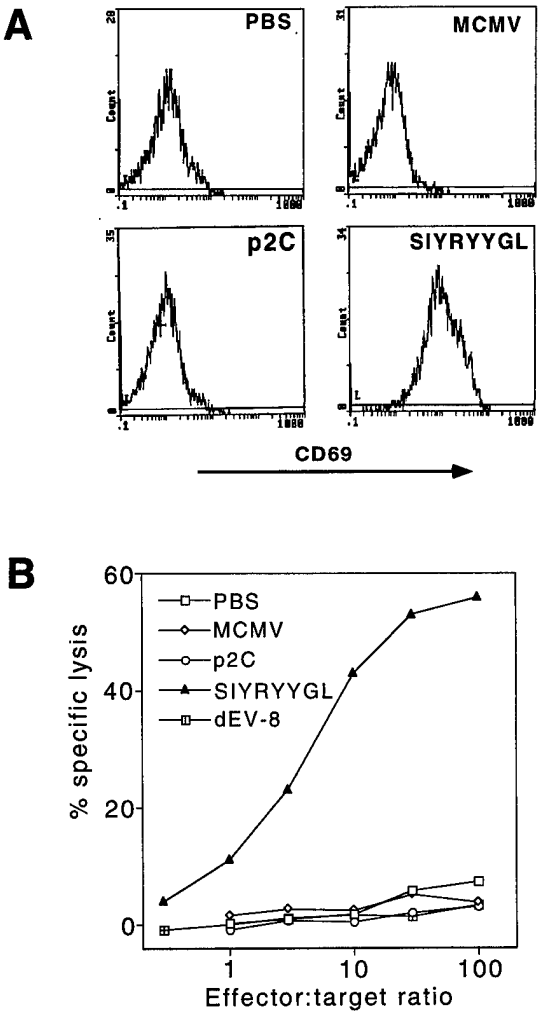

FIGURE 10. Peripheral activation of 2C T cells after injection of self-restricted peptide. *A*, Up-regulation of CD69 T cell activation marker on 1B2$^+$ T cells on day 3 following i.p. injection of antigenic peptide on days 1 and 2. Amounts of peptide injected for the figures shown are 70 nmol for MCMV and p2C and 10 nmol for SIYRYYGL. Injection of larger amounts of SIYRYYGL frequently caused death of the animals secondary to shock-like effects. Peptides were injected i.p. in 200 μl of PBS. *B*, Ex vivo cytolytic activity of splenocytes from mice injected with antigenic peptides in PBS. Spleens were harvested, RBCs were lysed, and splenocytes were assayed at various concentrations against ^{51}Cr-labeled P815 target cells in a standard 4-h assay.

signals to T cells under certain conditions (47, 48). It has been demonstrated that naive T cells from CD28-deficient, OVA-specific Th cell transgenic mice can initiate, but not sustain, an immune response (49). This system differs from our own in that other endogenous nontransgenic T cells are present, and the transgenic T cells are of the CD4$^+$ helper phenotype. Yet another alternate costimulatory pathway that could be substituting for B7:CD28 involves the CD58:CD2 interaction, which has been shown to elicit production of IFN-γ by CD8$^+$ T cells (50).

Each of these possible mechanisms is consistent with the observation that 2C T cells from the site of the tumor at the time of rejection express CD25 and are able to lyse the appropriate tumor cells ex vivo (Fig. 8 and Table I). The fact that the rejection of P815 appears to be independent of B7 does not suggest that B7 molecules are unimportant in the development of a CD8$^+$ CTL response, only that they are not critical for rejection in this system. It may well be that the 2C TCR/RAG mouse could reject much larger tumor burdens if the tumor was transfected with B7. Similarly, B7-positive tumors might be rejected by mice with much lower CTL precursor frequencies.

The fact that several of the TCR/RAG animals eventually succumbed to tumors at a very late stage (Fig. 3*A*) indicates that while tumor control is generally achieved, 2C CTLs did not eliminate the entire tumor load in at least some cases. This result is reminiscent of the persistence of viremia in CD4-deficient or B cell-deficient mice (40, 51, 52), providing support for the idea that CD4$^+$ T cells are important not only for initial CTL priming, but also to help sustain the CTL response (40). A recent report using purified CD8$^+$ 2C T cells has suggested that the maintenance of CTL effector function is dependent on either Th cell-derived cytokines or costimulatory ligands distinct from B7–1 (53). The high number of activated macrophages isolated from the peritoneum (Fig. 5*A*) implies that a source exists for the IFN-γ necessary for the activation of these cells (54, 55). The nature of this source is unknown, although T cells or NK cells could be providing it. The observation that recovered tumor cells and cultured P815 exhibited equivalent susceptibility to killing suggests that the 2C T cells from these animals have become exhausted or peripherally tolerized. In this

regard, the 2C TCR/RAG mice should provide a useful system for investigating $CD8^+$ T cell memory and tolerance.

We did not observe high levels of activation in splenic T cells obtained from mice during the period when rejection was occurring (Fig. 7 and data not shown). It is clear, however, from the in vivo response to SIYRYYGL that the CTL precursors in the TCR/RAG are indeed capable of full activation (Fig. 10). The effector response in the peritoneum of tumor-rejecting mice is characterized by an infiltrate consisting of both 2C CTLs and nonspecific effector cells. It is likely that the effector cell kinetics vary somewhat in both peak strength and timing between various mice. The fact that the lytic activity of the unfractionated PECs is about one-third peptide independent and about two-thirds peptide dependent (Table I) suggests that the 2C T cells may have initiated a Th1-like local response, recruiting and activating macrophages, neutrophils, and NK cells, which may all attack tumor cells. While macrophage-mediated tumor killing is typically measured in an 18- to 24-h assay, at least some of the peptide-independent ^{51}Cr release by unfractionated PECs may be attributable to this cell population. This is consistent with a recent report examining the role of Ag receptor-negative cells in the effector response to an adenocarcinoma (56). Moreover, such a Th1-like response (57) initiated by $CD8^+$ CTLs would explain the delayed onset of EL4 tumors in mice injected with a mixture of EL4 and P815 (Fig. 4). However, the ultimate failure to reject these tumors provides additional evidence for the critical role of the 2C CTLs in the development and maintenance of an antitumor response.

It has recently been demonstrated that CD28 costimulation promotes production of the Th2-like cytokines IL-4 and IL-5 by both $CD4^+$ and $CD8^+$ T cells (58). It is conceivable that presentation of Ag by B7-negative P815 to 2C pCTL might result in less Th2/Tc2 differentiation, allowing a skewing of the T cell response toward the Th1/Tc1 phenotype. Alternatively, another possible explanation for the significant levels of peptide-independent lysis seen by PECs during the period of rejection could rest in the recent observation that Th2-type $CD8^+$ effectors show extensive peptide-independent killing of target cells (59) and also differ from Th1-type $CD8^+$ effectors in their levels of Fas-mediated cytolysis (60). The high numbers of activated macrophages in the animal with tumor recrudescence suggests that a perhaps ineffective Th1-type response may have predominated in this particular mouse. It remains to be determined which of the two pathways of development (Th1- or Th2-like) is followed by naive 2C T cells when challenged with P815 in vivo.

It was previously reported that purified $CD8^+$ 2C CTLs could not proliferate in response to P815 (41). To reconcile our observation that 2C T cells do proliferate weakly in response to P815 with these previous results, it is important to realize that the number of stimulator cells in the assay can have a profound impact on whether proliferation is observed. At the highest densities used in this earlier work (2×10^5/well), we also observed similar low levels of proliferation. However, at lower stimulator cell densities, significant proliferation (up to 21,000 cpm) was detected even in the absence of exogenous cytokines (Fig. 9). This inverse effect of P815 cell density on the proliferative response of purified $CD8^+$ T cells was also described in a previous report (61). Moreover, it has recently been shown that the initial response to large doses of Ag does not require costimulation, but that costimulation plays a key role in prolonging the response (43). These findings are in keeping with the idea that costimulation up-regulates molecules that serve to protect against apoptotic death (11). In fact, the observation that tumors can reoccur in some mice suggests that the ability to mount a prolonged response may have been affected. We are currently examining this possibility.

Allograft rejection by TCR ($RAG^{+/+}$) mice has previously been examined (62), and it was found that female H-Y-specific TCR transgenic mice failed to reject male skin grafts. The authors suggested that this failure to reject might be attributed to a deficiency of $CD4^+$ Th cells secondary to skewing of the repertoire toward $CD8^+$ CTLs. In contrast, we found that the absence of $CD4^+$ cells does not prohibit rejection of allogeneic tumors. These results might be explained by differences in the type of allograft (i.e., skin- vs hemopoietic-derived tumor cells). In this respect, it will be of interest to examine the responses of 2C TCR/RAG mice to skin and cardiac allografts. Alternatively, the explanation may lie in differences in the overall avidity of the TCR/Ag interaction, taking into account both the high epitope density and the intrinsic TCR affinity of the 2C TCR/p2C/L^d interaction and the lower avidity of the H-Y/TCR interaction. Recent work has demonstrated that 2C TCR mice ($RAG^{+/+}$) will vigorously reject cardiac allografts in a situation where all three sources of help for a CTL response are possible (63). The density and load of allograft cells may well be critical in controlling the extent to which CTL precursors either become activated or become peripherally tolerized. It was recently reported that adoptive transfer of 2C T cells (both $CD8^+$ and $CD8^-$) into L^d-bearing SCID mice (where L^d is expressed on all nucleated cells) resulted in a tolerant state achieved through both activation-induced apoptosis and down-regulation of CD8 and the TCR (64). In contrast, when cardiac allografts were transplanted into 2C TCR transgenic mice ($RAG^{+/+}$) treated with rapamycin, T cells showed impaired intracellular signaling but unchanged surface levels of 1B2 and CD8 (63). Clearly, the density of L^d-expressing cells is much greater in an L^d SCID mouse than in the case of cardiac allografts or L^d-bearing tumor cells.

In conclusion, we have described a system in which allorejection occurs in the absence of $CD4^+$ Th cells and independent of costimulation through CD28-B7 interactions. The cell responsible for this allorejection is an MHC class I-restricted, $CD8^+$ CTL of a single specificity acting in the absence of $CD4^+$ cells. Our results suggest that these $CD8^+$ effectors may be mediating their effects at least partly by the induction of a Th1-like local response to the allogeneic tumor cells. These findings highlight the flexibility of the immune response and raise additional questions about an absolute requirement for $CD4^+$ cells in allorejection (7). In addition, the 2C TCR/RAG mice provide a useful system to develop and test agents such as bispecific Abs and other immunomodulating agents that can effectively redirect and/or prolong the activity of a CTL response against target cells.

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Targeting tumor cells with bispecific antibodies and T cells

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Abstract

It has been known for some time that mammalian immune systems are capable of eliminating large tumor burdens. Redirecting the immune response of a patient to an established tumor has now become the focus of various therapeutic strategies. In this report, two projects toward this goal are described. The first project involves the development of a transgenic mouse model for T cell directed therapeutics. These mice express specific T cell receptor α and β transgenes on a background in which the recombinational-activating-gene-1 (RAG) has been knocked out. The mice express cytotoxic T cells but not either T helper cells or B cells. Despite these deficiencies, the animals are capable of eliminating tumors that express the appropriate peptide/major histocompatibility complex ligand that is recognized by the αβ transgenic T cell receptor. Human tumors grow as transplants in these mice, thereby allowing various agents that redirect the endogenous T cells against human tumors to be tested. The second project involves a description of such agents: bispecific antibodies that simultaneously bind to an immune effector cell and a tumor cell. The bispecific antibody described here consists of folate attached to anti-T cell receptor antibodies, or their fragments. A single-chain Fv coupled with folate can redirect the lysis of human tumor cells that bear the high affinity folate receptor. Preliminary in vivo data showed that the folate/antibody conjugates were also capable of mediating rejection of the human tumor. This transgenic mouse model should now allow the evaluation and optimization of bispecific agents that can redirect a patient's own T cell response. © 1998 Elsevier Science B.V.

Keywords: Cytotoxic T Lymphocytes; Transgenic Mice; Bispecific Antibodies; Folate Receptor

1. Introduction

Most antibody-mediated targeting systems rely on the delivery of a linked, toxic compound in order to destroy the tumor. These approaches must overcome potential side effects associated with the toxic compound, either in the conjugate form or after release from the antibody. An alternative strategy, that has now been under investigation for the past 12 years, is to use the patients own T cells as the 'toxic' moiety. There are several potential advantages of using T cells in this capacity: (1) cytotoxic T lymphocytes (CTL) are present in large numbers in an individual; (2) each CTL has multiple mechanisms for killing a target cell, thereby limiting the outgrowth of tumor variants that might escape a single mechanism; (3) each CTL can kill multiple tumor targets; and (4) CTLs are a normal (i.e. self) component of an individual, and thus they are not recognized and cleared by the immune system, as might be the case with some toxins.

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One strategy to redirect the activity of T cells to a tumor has been the use of bispecific antibodies [1] that contain one antibody to a tumor antigen and another antibody to the T cell receptor complex (TCR). The bispecific antibody can mediate the lysis of the tumor cell by bridging CTL and tumor cell and by activating the CTL lytic machinery through the TCR complex. However, before a CTL can kill a tumor cell, it must be activated through simultaneous triggering of multiple cell surface molecules. There are now various strategies to accomplish such activation [2]. The goals of our work are to understand how a patients own CTLs might be most easily and effectively used, in conjunction with bispecific antibodies, as a therapeutic agent in cancer.

Two projects will be described in this report. The first involves the generation of a transgenic strain of mice (TCR/RAG) that will accept human tumor transplants, yet which contains endogenous CTLs. Thus, various drugs that can potentially be used for the activation and retargeting of CTLs to human tumors in vivo can be tested in these mice. Examples of the potency of tumor rejection by CTLs in these TCR/RAG mice will be shown. The second project involves the design and evaluation of the smallest bispecific antibodies yet produced, a conjugate of folate and an anti-TCR single-chain antibody (~30 kDa). These conjugates target the high affinity folate receptor (FR) present on most ovarian cancers and some brain tumors [3,4].

2. Materials and methods

2.1. Generation of transgenic mice

Mice that express αβ TCR trangenes from the CTL clone called 2C [5,6] were crossed with recombination activating gene-1 knockout mice (RAG−/−) [7]. Some T cells from the TCR transgenic mice express endogenous TCR and thus these mice contain T helper cells and they are capable of rejecting allogeneic and xenogeneic tumor transplants. In contrast, RAG−/− mice do not contain either B cells or T cells because they are not able to rearrange either Ig or TCR genes. The progeny of this cross and F1 backcrossed mice were examined for serum IgM and with the 2C TCR-specific mAb 1B2 [8] in order to develop a colony of TCR/RAG−/−mice. Serum IgM was monitored with an enzyme-linked immunoassay in which anti-kappa IgG coated on plates was used to capture serum Ig and bound IgM was detected with an HRP-labeled anti-mouse μ secondary antibody. To control for the total amount of serum added, various serum dilutions containing lysed red blood cells were analyzed for hemoglobin that was released, at an absorbance of 410 nm.

2.2. Tumor cell lines and transplantation

Tumor cell lines were used either as targets in cytotoxicity assays or in transplantation experiments with TCR/RAG mice. The following DBA/2 (H-2^d) derived tumor cell lines [9,10] were used as in vitro target cells: Mel, a murine erythroleukemia cell and F2-MTX^{r}A, a methotrexate resistant line selected for increased expression of FR by growth on low folic acid. The allogeneic mastocytoma line P815 (H-2^d), the syngeneic lymphoma EL-4 (H-2^b) and the human tumor cell lines SKOV-3, SKBR-3, BT474 (each erbB-2^+), and the FR$^+$ tumor KB [11] were transplanted into TCR/RAG mice and, in some cases, used as target cells in vitro.

Tumor cells, generally 10^7 for intraperitoneal experiments and 10^6 for subcutaneous experiments, were washed thoroughly in PBS and injected into either the peritoneal cavity or the shaved back of mice. For subcutaneous tumors, growth was assessed weekly or biweekly using calipers. Mean tumor diameter was calculated as the average of two perpendicular measurements. Animals were sacrificed when tumors reached diameters above 25 mm, or if the mice became overtly ill or the tumor became ulcerated. Mice injected i.p. were examined daily. Survival was assessed as either the day of death or the point when moribund mice were sacrificed. Observation of mice injected i.p. was continued through a ten week period.

2.3. Bispecific antibodies

The following monoclonal antibodies were used: 1B2, a mouse IgG1 specific for the TCR of CTL 2C [8] and KJ16, a rat IgG antibody specific for the Vβ8 region of the TCR [12]. KJ16 Fab fragments and KJ16 scFv were generated and purified as described

previously [13]. Briefly, scFv was refolded from inclusion bodies and monomeric scFv was purified by G-200 HPLC purification. Folate was coupled through its carboxyl groups to antibody amine groups using a carbodiimide procedure described previously [14,15]. Antibody conjugates were analyzed by mass spectrometry and absorption spectrophotometry.

2.4. In vitro CTL assays

The activity of CTL were examined in a cytotoxicity assay, in which tumor target cells were labeled with ^{51}Cr and effector cells were added with or without bispecific antibodies. Folate/antibody conjugates and cells were diluted in folate-free media and added to triplicate wells. Plates were incubated for 4 h and supernatants were removed for gamma counting. Cytotoxicity was determined by: % specific release=({experimental counts−spontaneous counts}/{maximal counts−spontaneous counts}× 100), where spontaneous release is measured in the absence of CTLs and maximal release is measured with detergent lysis.

3. Results and discussion

Results of the two projects involving tumor cell targeting with bispecific antibodies and T cells will be described. The first project is designed to evaluate the potency of CTL-mediated tumor rejection, by using a transgenic mice that express CTLs but not T helper cells or B cells. The second project describes an approach to redirecting the activity of CTL, using a novel form of bispecific antibody.

3.1. Transgenic mouse model for CTL-mediated tumor rejection

In the normal host response to tumor antigens, there is a complex interaction among immune cells that leads to the elimination of the tumor cell. This interaction is known to involve T helper cells, antigen presenting cells (e.g. B cells and macrophages), and CTLs. We wished to determine if CTLs alone are capable of eliminating tumor cells and, if so, what strategies might be optimum for redirecting their activity exclusively to a tumor burden. Furthermore, we wished to develop an animal model that would allow human tumors to be successfully transplanted but would also contain endogenous CTL for in vivo targeting studies (unlike nude and SCID mice which lack T cells).

To approach these questions, a strain of mice (TCR/RAG) was produced that contains only CTLs of a single TCR type and specificity (and which does not recognize any known human tumor antigens) (2) (Fig. 1). The TCR/RAG mice were produced by breeding a transgenic mouse that expresses the α and β chains from CTL clone 2C [5,6] and RAG-1 knockout mice [7]. The phenotype of adult TCR/RAG animals was examined in order to determine if they indeed lacked all B cells and T helper cells (i.e. CD4+ cells). Screening of offspring of different ages, from matings of TCR tg mice with RAG−/− mice, was performed by flow cytometry of spleen cells. These results revealed that essentially all of the splenic T cells are $CD8^+$ (i.e. CTLs) (Table 1) and that they all express the same TCR as identified with an anti-TCR antibody, 1B2 [8]. TCR/RAG mice exhibited, on average, slightly reduced spleen and thymus sizes compared to normal mice or TCR transgenic mice (Table 1 and data not shown). The mice also lack any B cells, as evidenced by the lack of serum IgM (Fig. 2).

Despite the presence of only CTLs in the TCR/

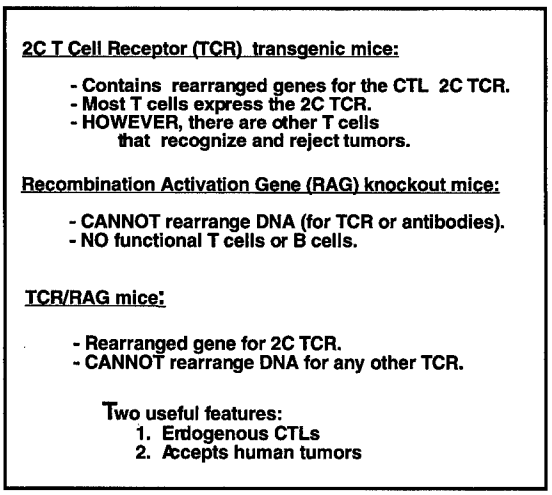

Fig. 1. Features of the 2C CTL TCR transgenic mice, RAG-1 knockout mice, and the $TCR/RAG^{-/-}$ progeny.

Table 1
Flow cytometric analysis of splenic lymphocyte populations in TCR/RAG-1$^{-/-}$ mice

Strain/Age	Sex	Percent of T cells[a]			Spleen wt (mg)	Cell count ($\times 10^7$)
		CD4+8−	CD4+8+	CD4−8+		
C57/B6						
5 mo	F	66	0	33	80	5
5 mo	F	55	0	45	82	6
5 mo	M	63	0	37	228	
2C TCR						
5 mo	F	31	0	72	107	
TCRRAG−/−						
1 mo	F	1	1	98	36	.6
1 mo	M	4	2	94	80	1.7
2 mo	F	1	1	98	92	3.2
2 mo	F	1	1	98	54	2.2
2 mo	M	2	1	97	52	1.1
2 mo	M	1	1	98	42	
3 mo	M	1	1	98	113	
3 mo	M	5	3	93	98	
4 mo	M	2	0	98	47	1.9
4 mo	M	1	0	99		
5 mo	M	1	2	96	75	
5 mo	M	1	0	99	144	
5 mo	F	0	2	98	91	
5 mo	F	0	1	99	58	
6 mo	M	5	0	95	48	
6 mo	F	1	0	99	186	
TCRRAG−/−	median	1	1	98	75	

[a]Total T cells was calculated as (CD4+8−)+(CD4+8+)+(CD4−8+).

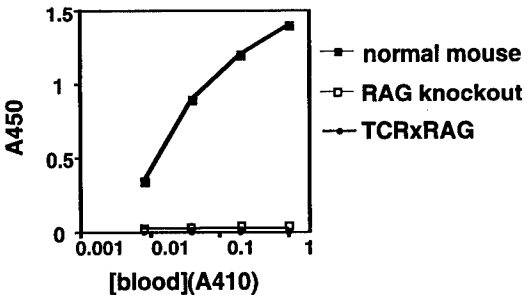

Fig. 2. ELISA of serum IgM from a normal C57Bl/6 mouse, a RAG-1$^{-/-}$ mouse, and a TCR/RAG$^{-/-}$ mouse. Blood was lysed by hypotonic shock and diluted for analysis in 96-well plates. Absorption at 410 nm of these samples was monitored immediately, prior to completion of the ELISA, as a measure of the amount of hemoglobin (i.e. directly proportional to the amount of blood in the sample). After these measurements were made, a standard ELISA for serum IgM was performed using anti-IgM specific antibodies, as described in Section 2.

RAG mice, CTLs could be activated both in vitro and in vivo with appropriate stimulating antigens. The transgenic TCR recognizes a peptide p2C (LSPFPFDL) bound to the class I MHC L^d [16] or a peptide SIYRYYGL bound to the class I MHC K^b [17]. Spleen cells from TCR/RAG mice injected with the SIYRYYGL peptide or spleen cells that were cultured with either SIYRYYGL/K^b or p2C/L^d proliferated and were shown to be activated by various criteria: expression of IL-2 receptors, expression of the CD69 antigen, and their ability to kill tumor cells that bear these antigens (Ref. Manning et al. submitted for publication).

In addition, the TCR/RAG mice were fully capable of eliminating tumor cells that bear the appropriate peptide/MHC ligand recognized by the tg TCR. The p2C/L^d complex is expressed by the DBA/2 tumor P815, but not by various other tumor cell lines. To investigate whether the TCR/RAG could reject this allogeneic tumor load, P815 cells

were injected either subcutaneously or intraperitoneally. The TCR/RAG animals injected with 10^7 cells showed no signs of illness and with one exception, survived without event (Fig. 3). Control RAG−/− animals failed to reject the allogeneic tumors and died or became moribund within three weeks. To examine the strength of the response, mice were injected with a 30-fold greater tumor load (3×10^8 P815 cells). Two of three high tumor load animals survived through the six week experimental period without event, and one succumbed to its tumor late in the course at day 42. The observation that tumors are rejected by the TCR/RAG animals and not rejected in the RAG mice indicates that the transgenic $CD8^+$ CTL is the cell type responsible for the rejection. In addition, TCR/RAG mice failed to reject other mouse tumors such as EL4, that do not express the appropriate ligand recognized by the transgenic CTL (Fig. 4). This result further shows that peptide/L^d-specific CTL of the TCR/RAG mice are required for rejection.

Finally, various human tumor cell lines were transplanted into TCR/RAG mice in order to determine if they developed tumors and thus could be used as models for drugs that target CTLs to tumors. The erbB-2^+ tumors SKOV3 and BT474 (but not SKBR-3) and the high affinity FR^+ tumor line KB all grew as subcutaneous tumors (Fig. 4 and data not shown). Because human tumor cells are not killed by activated 2C CTLs, we expected that the KB tumor would grow in TCR/RAG mice even when endogenous T cells were activated by administration of the SIYRYYGL peptide. This expectation was met, as the KB tumor grew equally well in peptide-treated and untreated mice (Fig. 4). Thus, the KB tumor line can serve as a model for targeting of activated CTL, using folate/anti-TCR conjugates described below.

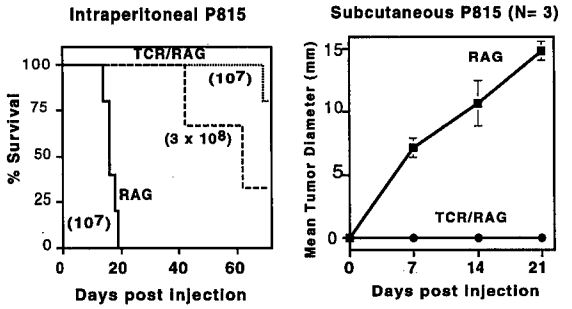

Fig. 3. Tumor allograft rejection in TCR/RAG mice. In the panel on the left, TCR/$RAG^{-/-}$ mice were injected i.p. with either 10^7 P815 cells (n=5 mice) or 3×10^8 P815 cells (n=3 mice). RAG-$1^{-/-}$ mice received injections of 10^7 P815 cells (n=5 mice). Animals were monitored as described in Section 2 for a period of ten weeks. In the panel on the right, RAG-$1^{-/-}$ or TCR/$RAG^{-/-}$ mice (n=3) were injected s.c. in the shaved back with 10^6 P815 cells in 100 μl and monitored weekly for tumor growth. Mean tumor diameter represents the average of two perpendicular measurements.

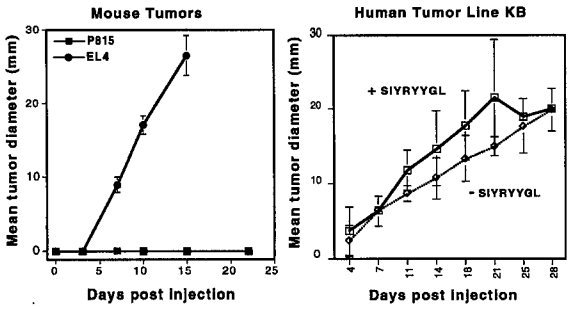

Fig. 4. TCR/$RAG^{-/-}$ mice do not reject tumors that lack the appropriate peptide/MHC ligand. In the panel on the left, TCR/RAG mice were injected s.c. with 10^6 EL4 (H-2^b) or P815 (H-2^d) in the shaved back in 100 μl of PBS. In the panel on the right, TCR/$RAG^{-/-}$ mice were injected s.c. with 3×10^6 human KB tumor cells. Mice received either 10 nmoles peptide SIYRYYGL (+SIYRYYGL) or saline (−SIYRYYGL) 2 days prior to tumor transplantation. N=3 for all experiments. The absence of error bars indicates a standard deviation smaller than the size of the symbol.

3.2. *Characterization of bispecific folate/antibody conjugates*

Bispecific antibodies that bind to a tumor antigen and the TCR redirect CTLs to lyse tumor cells which have escaped normal immune recognition mechanisms. One well-characterized tumor antigen, the FR, is expressed on most ovarian carcinomas. Recently, we showed that conjugates of folate and anti-TCR antibodies are extremely potent bispecific agents that target tumor cells expressing the high affinity folate receptor, but not normal cells expressing only the reduced folate carrier protein [18].

The size of these conjugates can be reduced to the smallest bispecific agent yet described (30 kDa) by attaching folate to a single-chain antibody, scFv, of the anti-TCR antibody called KJ16 (Fig. 5). Folate was attached through a carboxyl group to scFv

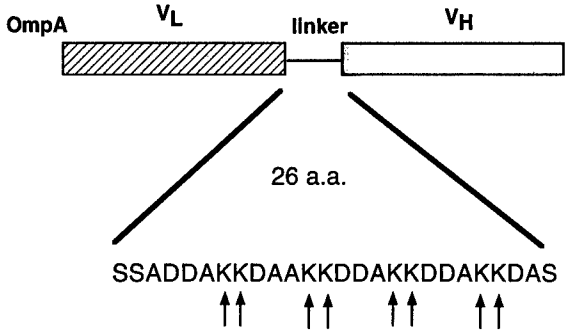

Fig. 5. Diagram of the single-chain Fv from the anti-T cell receptor antibody KJ16. The 26 amino acid linker that contains multiple lysine residues for carbodiimide coupling of folate is shown.

amines using a carbodiimide reaction. The scFv/folate conjugates were as effective as intact IgG/folate conjugates in mediating lysis of FR^+ tumor cells by CTL (Fig. 6) [19]. The optimal folate density was in the range of 5 to 15 folate molecules per scFv or IgG molecule, which yielded EC_{50} values of approximately 40 pM (1.2 ng ml^{-1} for scFv). The scFv/folate conjugates could also efficiently target tumor cells in vitro even in the presence of free folic acid at concentrations that are normally found in serum [19].

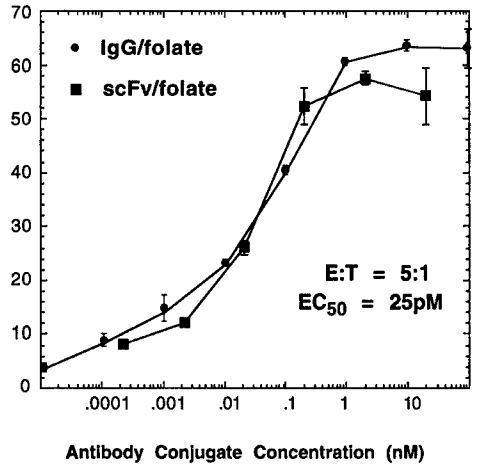

Fig. 6. Comparison of scFv/folate and IgG/folate in CTL-mediated lysis of tumor cells. Folate was conjugated to KJ16 scFv and intact antibody under identical conditions (3.3 μM EDC, 100:1 molar ratio of folate:antibody) yielding conjugates with 7 fol/scFv (●) and 5 fol/IgG (■). Cytotoxicity assays were performed with these conjugates using ^{51}Cr-labeled F2-MTX^{r}A cells and CTL clone 2C.

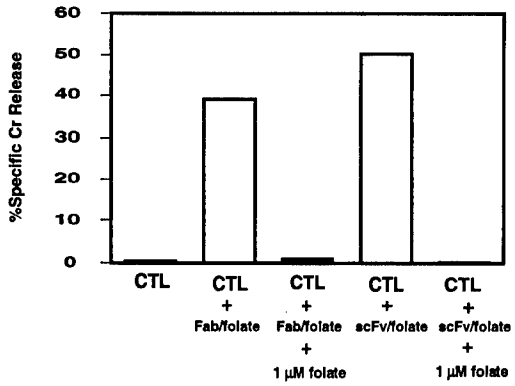

Fig. 7. Folate/antibody conjugates mediate lysis of FR^+ human tumor cell line KB by CTLs. Activated CTL from TCR/RAG mice were added to ^{51}Cr-labeled KB cells in the presence of the indicated agents. KJ16 Fab/folate and KJ16 scFv/folate preparations were each assayed at ~10 μg ml^{-1}. Free folate was added at 1 μM.

In vitro-activated CTLs from the TCR/RAG mice described above were able to kill the human FR+ tumor line KB in the presence of folate/scFv and folate/Fab conjugates (Fig. 7). This redirected lysis was completely inhibited by excess free folate and thus the effect is mediated through the high affinity FR.

Finally, a preliminary study has shown that a Fab/folate conjugate was capable of mediating the rejection of KB tumor cells in TCR/RAG mice that had been injected with SIYRYYGL peptide to activate CTL (Fig. 8). In this experiment, mice were co-injected with 3 million KB cells and 20 μg of the folate/Fab conjugate subcutaneously. Studies to further evaluate the in vivo targeting efficiency of the folate/scFv and folate/Fab conjugates will focus on several issues: (1) methods of in vivo CTL-activation; (2) route and timing of antibody delivery; and (3) optimization of the effective concentration and forms of the conjugates.

4. Conclusion

Using a transgenic animal model, we have demonstrated that CTL alone are potent mediators of tumor

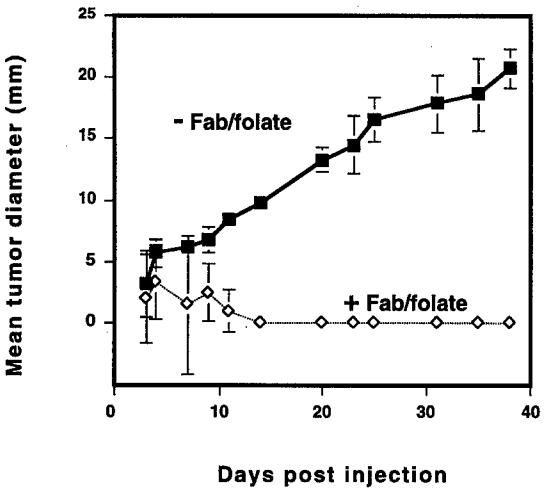

Fig. 8. Fab/folate conjugates mediate the rejection of KB tumor cells in TCR/RAG mice. TCR/RAG mice were injected s.c. with 3×10^6 human KB tumor cells in the presence (+) or absence (−) of 20 μg Fab KJ16/folate. Mice received 10 nmoles peptide SIYRYYGL, two days prior to tumor transplantation. $N=3$ for all experiments. The absence of error bars indicates a standard deviation smaller than the size of the symbol.

rejection. This finding suggests that it will be possible to focus strategies on the in vivo activation and retargeting of CTL to specific tumor cell antigens. In addition, the TCR/RAG mice can accept human tumor xenografts and thus they should serve as a useful model for the testing of bispecific agents that target human tumors.

In an effort to generate smaller and more potent bispecific agents, we have explored the use of folate/anti-TCR conjugates for targeting the high affinity FR, a tumor associated antigen present on most ovarian tumors. Folate directly coupled to a single chain V_LV_H antibody yielded a potent tumor targeting agent in cytotoxicity assays with CTLs. Compared to conventional bispecific antibodies, the small size of scFv/folate conjugates may prove advantageous in the ability to penetrate tumors, in reduced immunogenicity, and in eliminating Fc-mediated side effects.

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TARGETING T CELLS AGAINST BRAIN TUMORS WITH A BISPECIFIC LIGAND-ANTIBODY CONJUGATE

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High-affinity receptors expressed on the surface of some tumors can be exploited by chemically conjugating the ligand for the receptor and an antibody against immune effector cells, thus redirecting their cytolytic potential against the tumor. Ovarian carcinomas and some brain tumors express the high-affinity folate receptor (FR). In this report, a transgenic mouse model that generates endogenously arising choroid plexus tumors was used to show that folate/anti-T-cell receptor antibody conjugates can direct infiltration of T cells into solid brain tumor masses. An engineered single-chain Fv form of the anti-T-cell receptor antibody KJ16 was conjugated with folate, to produce a bispecific agent that was substantially smaller than most previously characterized bispecific antibodies. Folate conjugation to the antibody increased T-cell infiltration into the tumors by 10- to 20-fold, and significantly prolonged survival of the mice. *Int. J. Cancer* 76:761–766, 1998.

Tumors use a variety of mechanisms to avoid being eliminated by the immune system. Some tumors actively suppress immune function by secreting inhibitory cytokines or by killing T cells through Fas/FasL interaction. Other tumors escape recognition by cells of the immune system. For example, tumors may fail to present peptide antigens in complex with a product of the major histocompatibility complex (MHC) that are essential for recognition by T cells. Tumor cells may also be deficient in co-stimulatory ligands and adhesion molecules that facilitate recognition and T-cell activation. Finally, the early development of tumors may allow the immune system to become tolerant of potential surface antigens on tumors.

One potential way to overcome escape due to failures of recognition is to redirect immune cells against tumor cells with bispecific antibodies. Bispecific antibodies can be constructed to recognize 2 separate antigens, one on the tumor surface and the other on the surface of an immune effector such as a cytotoxic T cell. Many tumor cells have potential target antigens that are tumor specific or quantitatively more abundant on tumor cells than normal cells (tumor associated). Previous work has demonstrated the *in vitro* and *in vivo* effectiveness of bispecific antibodies against a variety of experimental tumors. Several clinical trials have been conducted with first-generation bispecific agents with results that are sufficiently promising to warrant further study of this strategy (Canevari *et al.,* 1995).

Despite considerable progress in the design of bispecific antibodies, significant obstacles to their effective clinical use remain. One of the problems associated with the bispecific antibody approach has been the difficulty in identifying animal models that can mimic human cancers. By analogy with human use, one would like a system that could evaluate how an endogenously arising tumor could be controlled by treatment with bispecific agents that redirect the activity of endogenous T cells. To date, all pre-clinical bispecific antibody studies have used transplanted tumors in either syngeneic rodent systems or xenogeneic systems in which human tumors and lymphocytes are transplanted in immunodeficient mice. This report shows that a new class of bispecific antibody agents can be used in a transgenic, endogenous tumor model to study T-cell-mediated therapies.

The tumor antigen targeted in this study was the high-affinity folate receptor (FR), which has been identified on ovarian carcinomas and most choroid plexus tumors and ependymomas (Mantovani *et al.,* 1994; Weitman *et al.,* 1992). The high-affinity FRs (K_D approx. 1 nM) differ from the ubiquitous lower affinity reduced folate carriers (K_D approx. 100 μM) that are largely responsible for normal folate uptake (Westerhof *et al.,* 1991). High-affinity FRs were originally identified as tumor-associated antigens using monoclonal antibodies that reacted with ovarian tumor cell lines (Coney *et al.,* 1991). The presence of FR on ovarian tumors has led to its use as a target for various forms of therapy, including bispecific antibodies (Mezzanzanica *et al.,* 1991).

The nanomolar affinity of folate for FR suggested that attachment of folate directly to an anti-T-cell receptor (TCR) antibody might be a rapid method to generate bispecific antibodies that efficiently target FR-positive tumor cells for lysis by activated T cells. In cytolytic assays, these folate/antibody conjugates have very potent targeting activity without adversely affecting normal cells that express only the reduced folate carrier protein (Kranz *et al.,* 1995). To reduce the size of the bispecific agent, we have produced folate conjugates of the single-chain Fv of the anti-Vβ8 antibody KJ16 (scFv KJ16), and the folate/scFv conjugates were as effective as the folate/IgG conjugates in cytotoxicity assays against FR-positive cells (Cho *et al.,* 1997). The 30 kDa folate/scFv conjugate is to our knowledge the smallest bispecific antibody yet reported.

To develop an animal model for testing the folate/bispecific agents, we chose SV11 mice that are transgenic for the SV40 large T antigen gene with the SV40 enhancer (Van Dyke *et al.,* 1985). SV11 mice develop choroid plexus tumors with 100% penetrance and in a well-defined time period (age of mortality averages about 100 days). SV40-induced choroid plexus tumors express FR with properties that are very similar to the human FR, including a K_D of 1 nM (Patrick *et al.,* 1997). In addition, flow cytometry and immunohistochemistry of FR on tumor cells indicated that virtually all the viable cells are FR positive (data not shown).

MATERIAL AND METHODS

Purification of scFv-KJ16

scFv-KJ16 was solubilized from *E. coli* inclusion bodies and refolded by dilution into 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM $CaCl_2$, 1 mM EDTA, 5 mM phenylmethylsulfonylchloride, as described previously (Cho *et al.,* 1995). After concentration by tangential flow, the scFv preparation was dialyzed into 20 mM Tris, pH 8.0, and purified by anion exchange chromatography using Q Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) eluting with 20 mM Tris, pH 8.0, 1 M NaCl. Peak fractions were pooled, concentrated, and dialyzed into PBS if used *in vivo* or 0.1 M

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MOPS, pH 7.5, if used for folate conjugation. The purity and activity of the KJ16 scFv preparations were assessed using a competition flow cytometry assay as described previously (Cho *et al.,* 1995). scFv-KJ16 purified in this manner had an affinity for TCR as reported previously (approx. 120 nM) and was approx. 95% monomeric as determined by gel filtration (G200).

Preparation of folate/scFv-KJ16 conjugates

Folate scFv conjugates were prepared and characterized as described previously (Cho *et al.,* 1997). Briefly, folate and the crosslinker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC, Pierce, Rockford, IL) were dissolved in DMSO at a 2.5 molar excess of EDC. After 30 min at room temperature in the dark, a 100-fold molar excess of the activated folate was added to the scFv-KJ16 previously dialyzed into 0.1 M MOPS. After 1 hr at room temperature, the sample was applied directly to a G-25 column pre-equilibrated in 0.1 M MOPS, pH 7.5. Fractions were characterized by absorbance at 280 and 363 nm, and peak fractions were pooled, dialyzed into PBS and filter sterilized. The biological activity of each preparation of the folate/scFv-KJ16 conjugate was tested in cytotoxicity assays before use *in vivo.* Folate/scFv-KJ16 conjugate preparations averaged 10 folate molecules per antibody molecule.

Animals

The SV11 strain was obtained from Dr. T. Van Dyke (University of North Carolina, Chapel Hill, NC). The strain is maintained heterozygous for SV40 large T antigen by mating transgene-positive males with C57BL/6J females and determining genotype of offspring by PCR for large T antigen. Transgene-positive males and females have the same survival curves.

In vivo *infiltration of T cells assessed by immunohistochemistry of CD3*

Control mice received 2 injections of PBS or staphylococcal enterotoxin B (SEB, Toxin Technologies, Miami, FL) (50 μg) followed by PBS. Experimental mice were injected with SEB and scFv-KJ16 without folate (10 μg, i.v., 18 hr after SEB), or SEB and folate/scFv-KJ16 conjugate (10 μg, i.v., 18 hr after SEB) and sacrificed after 1 or 2 days, at 93–95 days of age. Mice receiving SEB and IgG KJ16 or folate/IgG-KJ16 (10 μg) were sacrificed after 2 days. Tissues were fixed by perfusion with acetic acid/zinc/formalin (Newcomer Supply, Middleton, WI); the brains were then blocked into 2 mm thick pieces and paraffin embedded the next day by the University of Illinois College of Veterinary Medicine Histopathology Laboratory. Six blocks per slide were mounted together, allowing estimation of the total number of infiltrating T cells in a tumor. Three micron serial sections were deparaffinized, rehydrated to PBS, blocked with normal goat serum and incubated overnight at 4°C with primary antibody. The primary antibody was rabbit anti-CD3 (Dako, Carpinteria, CA), followed by biotinylated goat anti-rabbit secondary antibody (Vector, Burlingame, CA), avidin-biotin-HRP complex (Vector Elite ABC) and nickel-cobalt-enhanced diaminobenzidine (DAB; Pierce) for detection. Slides were counterstained with methyl green, dehydrated and coverslipped. Tumor areas were determined using NIH Image at 25×. Individual T cells were counted at 100× in 6 planes per brain; the total number of T cells in 6 planes ranged from 1 (a PBS-treated mouse) to 2,343 (a folate/scFv-KJ16 treated mouse). The number of mice in each condition was as follows: PBS (3), SEB (2), scFv-KJ16 at 24 hr (2), folate/scFv-KJ16 at 24 hr (4), scFv-KJ16 at 48 hr (4), folate/scFv-KJ16 at 48 hr (4), IgG-KJ16 at 48 hr (1) and folate/scFv-KJ16 at 48 hr (2).

In vivo *treatment to test survival benefit*

Experimental mice were placed on a low-folate diet at age 70 days to reduce serum folate levels. Commercial rodent diet contains high folate concentrations, and a reduced folate diet brings serum folate levels down to levels observed in humans (Mathias *et al.,* 1996). We measured serum folate levels in C57BL/6 mice fed a normal diet and a reduced folate diet and observed a reduction from 236 ± 12 nM to 56 ± 14 nM within a week, followed by relatively stable low levels (35 ± 3 nM at 4 weeks). In control experiments with n = 8 we found that neither the maintenance on low-folate diet nor the stress of repeated restraint and injection of PBS affected survival (data not shown). Baseline body weight of mice was obtained at 84 days. With previous cohorts of mice we correlated the loss of body weight due to cachexia and age of death to provide an objective criterion for morbidity. Mice died within 2–3 days of reaching 75% of baseline body weight, so this criterion was used to determine time of sacrifice; mice were also sacrificed if they showed clear neurological symptoms such as vestibular problems (from 4th ventricle tumors) or ataxia and lethargy.

Antibody-treated mice were injected i.p. with 100 μg SEB on day 84, followed 18 hr later by i.p. injection of either folate/scFv-KJ16 (25 μg in PBS, n = 8) or scFv-KJ16 (25 μg, n = 8). Mice received 3 additional i.p. injections of antibody (25 μg) at 4 day intervals. A single injection of SEB was employed to avoid SEB-induced anergy (Sabapathy *et al.,* 1994). Control mice (n = 9) were untreated. Mice were weighed and monitored daily for neurological signs.

Statistical analysis

Infiltration data were analyzed by analysis of variance using SAS JMP software. Survival data were analyzed by Kaplan-Meier estimates and the log-rank test, using SAS JMP.

RESULTS

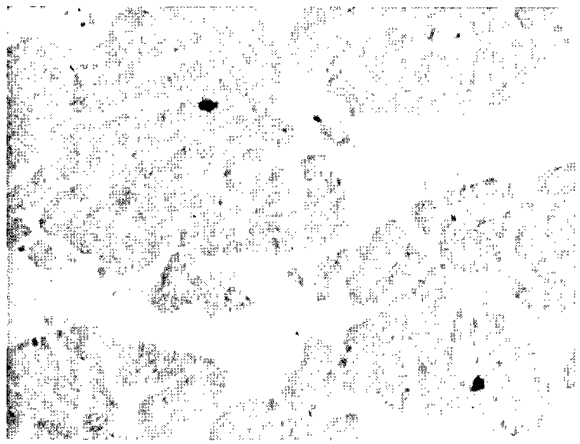
To determine whether the folate moiety of the bispecific conjugate could specifically target T cells against SV11 tumors, mice were injected with either scFv-KJ16 or folate/scFv-KJ16 preparations. T cells were activated 1 day before the injection of the bispecific agent by injecting the superantigen SEB, which activates the Vβ8 population of T cells recognized by KJ16. Mice were sacrificed 24 or 48 hr after antibody injection, and the number of T cells that infiltrated the tumor was evaluated by immunohistochemistry of CD3. In the absence of SEB or bispecific antibody, very few T cells were observed (as few as 1 or 2 per brain). SEB alone produced low levels of infiltration (Fig. 1*a*). Similarly, scFv-KJ16 without folate-conjugated antibody produced very low levels of T-cell infiltration (Fig. 1*b*). In contrast, treatment with the folate/scFv-KJ16 conjugate caused a substantial infiltration of T cells into the tumors (Fig. 1*c*). Quantification of T-cell density in the tumors confirmed that folate significantly increased T-cell infiltration both 24 and 48 hr following treatment ($p < 0.0001$, folate/scFv-KJ16 *vs.* scFv-KJ16, Fig. 2). The IgG form of folate/KJ16 produced a moderate level of T-cell infiltration at 48 hr but less than the single-chain form of the conjugate at the same time point (Fig. 2, $p = 0.05$).

Having demonstrated that the folate-conjugated antibody targeted T cells to the tumor, we then asked whether the folate/single-chain antibody conjugate was capable of redirecting a sufficient number of T cells to have an impact on survival. Minor modifications of the treatment protocol were made to optimize chances of improving survival. The animals were put on a low-folate diet to reduce circulating serum levels of folate that could compete with the bispecific agent, and multiple injections of a higher dose of folate/scFv-KJ16 were used.

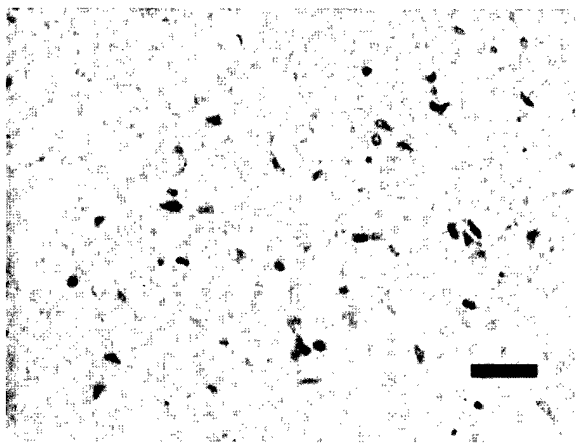
Our colony of SV11 mice has an essentially identical survival curve to the original description of the strain over 30 generations ago, with survival times of 100–105 days for various cohorts. To estimate when to begin treatment, we characterized the histological development of tumors in untreated animals. Consistent with an earlier description (Van Dyke *et al.,* 1987), a small number of hyperplastic foci usually begin to appear between 70 and 80 days of age, and then tumors become increasingly anaplastic and grow approximately exponentially over the next 3–4 weeks (Fig. 3).

Based on this characterization of tumor development, mice were treated beginning at day 84 with SEB and either scFv-KJ16 or

SEB



sc KJ16



sc KJ16 Folate Conjugate

FIGURE 1 – Infiltration of choroid plexus tumor by T cells following treatment with folate/anti-TCR bispecific antibody. (*a*) Immunohistochemistry of T-cell marker CD3 following treatment of SV11 mice with SEB (50 μg i.p.). Tumor cells are counterstained with methyl green. (*b*) Immunohistochemistry of T-cell marker CD3 following treatment of SV11 mice with SEB (50 μg i.p.) and scFv-KJ16 antibody (10 μg i.v.) reveals minimal T-cell infiltration when folate is not conjugated to the antibody. (*c*) Folate/scFv-KJ16. Immunohistochemistry of CD3 following treatment with SEB (50 μg i.p.) and folate/scFv-KJ16 antibody (10 μg i.v.). Extensive infiltration of darkly staining T cells is observed. Scale bar = 50 μm.

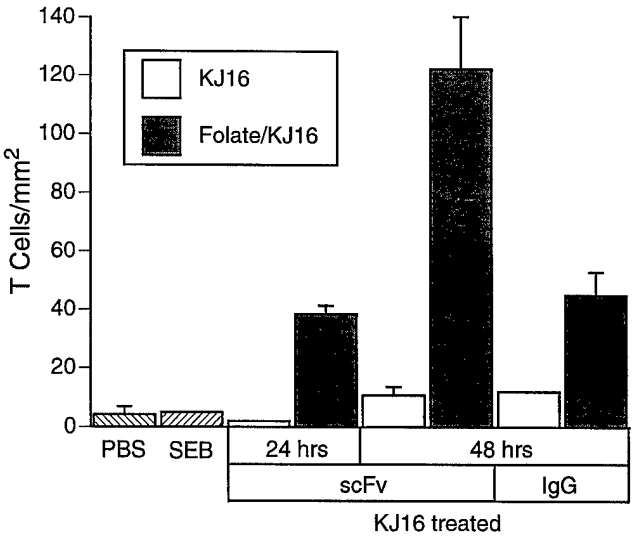


FIGURE 2 – Quantitation of T cell density in tumors following treatment with various anti-TCR antibody preparations. Controls were injected with PBS vehicle or superantigen SEB. Experimental mice were injected with SEB followed by 1 of 2 forms of the anti-Vβ8 TCR antibody KJ16, either single-chain Fv (scFv) or IgG. Each of the 2 forms of KJ16 was either conjugated with folate or non-conjugated. Mice receiving the scFv antibodies were sacrificed 24 or 48 hr following antibody treatment, and mice receiving IgG antibodies were sacrificed 48 hr following antibody treatment. Conjugation of the antibody with folate increased the infiltration of T cells into the tumor in all cases, with the highest density of T cells observed 48 hr following the scFv form of the folate conjugate.

folate/scFv-KJ16. A single dose of SEB was followed 18 hr later by an injection of antibody. Three additional injections of antibody were given over the next 12 days. Treatment with SEB and scFv-KJ16 resulted in a significant prolongation of survival compared with untreated controls ($p < 0.05$). However, treatment with SEB and folate/scFv-KJ16 resulted in a further significant enhancement of survival ($p < 0.05$ compared with scFv-KJ16, Fig. 4). Survival times were 100 days for controls, 112 days for scFv-KJ16 treated mice and 120 days for folate/scFv-KJ16 treated mice.

DISCUSSION

We have demonstrated that endogenous T cells can be redirected to infiltrate and attack brain tumors. The action of T cells was sufficient to roughly double the survival time following the initial development of tumors in SV11 mice (*i.e.,* approx. 20 days from day 80 to 100 for untreated mice compared with approx. 40 days from day 80 to 120 for treated mice). Previous reports have demonstrated tumor infiltrating lymphocytes in established solid tumors after bispecific antibody treatment (Thibault *et al.,* 1996), but few have demonstrated a reduction in solid tumor growth or prolongation of survival (Kroesen *et al.,* 1995). The present findings are noteworthy given the difficulties posed by the blood-brain barrier and the relative immune privilege of the brain. The transgenic nature of the model makes it additionally challenging to treat; because of the expression of SV40 large T antigen in every choroid plexus cell, each is a potential new focus of tumor development. Thus, even if the existing tumors were completely eradicated, new tumors would presumably arise. It may therefore be necessary to develop a full memory response in T cells to cure tumors in this particular model.

Activation of T cells by SEB appeared to have a beneficial effect on survival, as mice treated with SEB/unconjugated scFv-KJ16

lived significantly longer than control mice. This finding is consistent with earlier reports that activation of T cells by SEB or an anti-CD3 antibody such as 145-2C11 can generate anti-tumor activity in mice (Newell *et al.*, 1991; Penna *et al.*, 1994). In a separate trial, we treated SV11 mice with 145-2C11 and produced a survival benefit similar to that in mice treated with SEB/unconjugated scFv-KJ16 in the present experiment (data not shown). We do not know why in both of these cases there was anti-tumor activity from these treatments in the absence of substantial T-cell infiltration, at least at early time points after the treatment (Fig. 1*a* and data not shown for 145-2C11). It may be relevant that for the present survival study that animals received twice the dose of SEB as the T-cell infiltration study. However, in earlier *in vivo* studies of SEB alone against murine tumors, doses ranging from 50 to 250 μg/mouse produced similar anti-tumor activity (Newell *et al.*, 1991; Penna *et al.*, 1994).

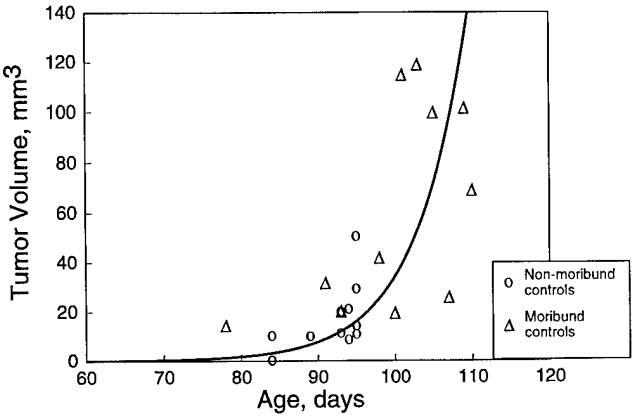


FIGURE 3 – Development of tumors in SV11 mice. Mice were sacrificed at various ages, either while still healthy (non-moribund, circles) or moribund by neurological or body weight criteria (triangles). Tumor volume was calculated from tumor area, determined with NIH Image from evenly spaced sections throughout the brain. Tumors develop rapidly after approximately day 80.

In the present study, we did not test the effect of the bispecific conjugate in mice that did not have T cells activated by SEB, but in another murine model (a transplant model of an FR-positive tumor), activation of T cells greatly enhanced the effect of the same antibody/conjugate (data not shown). Similarly, Penna *et al.* (1994) observed less effect of a bispecific antibody against melanoma cells when no *in vivo* activation with SEB was employed.

Our results emphasize the importance of both ligand-based targeting of tumors and the use of the smaller scFv form for systemic treatment of solid tumors. The folate conjugate of the scFv form of KJ16 elicited a substantial increase in T-cell infiltration and resulted in enhanced survival that was significantly greater than the scFv without folate. For example, there was an approx. 20-fold increase in T-cell number at 24 hr after treatment with folate/scFv-KJ16 and an approx. 10-fold increase in T-cell number at 48 hr after treatment compared with scFv-KJ16 without folate. The single-chain form of the antibody also appeared to be important, as the folate/scFv-KJ16 produced an approximately 3-fold higher number of T cells compared with the IgG form of the folate/KJ16 conjugate at 48 hr. However, we did not conduct a pharmacokinetic analysis of the 2 forms of antibody/conjugate, and the difference between single-chain and IgG forms may be a difference in time course. In previous comparisons of IgG and single-chain antibodies, the single-chain antibody has also provided better access of the antibody to T cells or better tumor penetration from systemic circulation (Yokota *et al.*, 1992). These measurements provide a useful guide to the possible relationship between the extent of T-cell infiltration into a tumor and the benefit regarding survival. In the present experiments, the animals investigated for T-cell infiltration were not on a low-folate diet, whereas the animals in the survival experiment were on a low-folate diet, so the actual number of T cells in the latter case may have been higher than we calculated. Extensions of these studies should allow us to

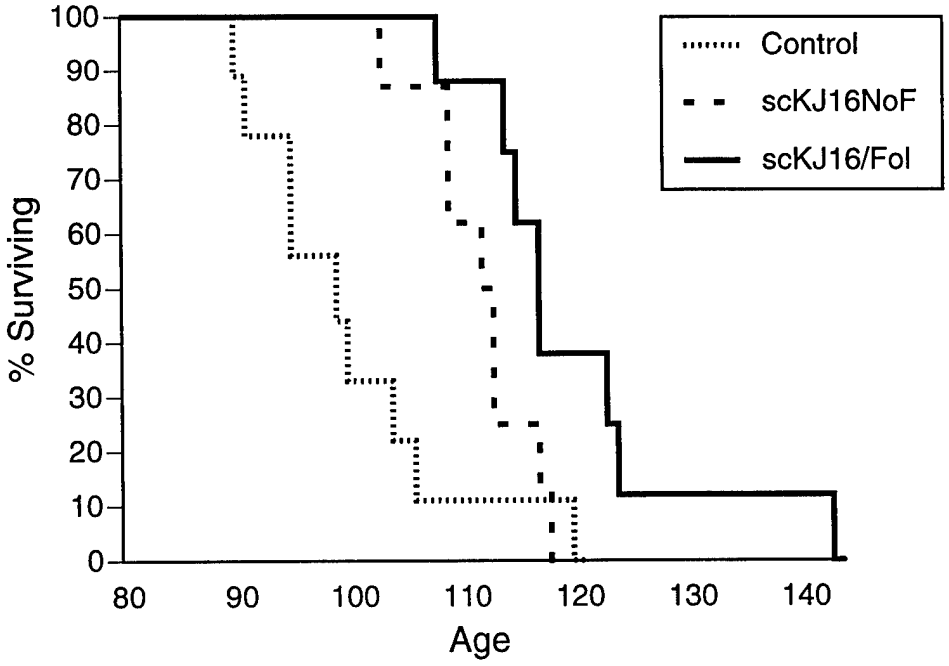


FIGURE 4 – Prolongation of survival of SV11 mice following antibody treatments. Experimental mice were treated with SEB (100 μg i.p.) at day 84 followed over the next 13 days by 4 treatments with either scFv-KJ16 (25 μg i.p.) or folate/scFv-KJ16 (25 μg i.p.). Mice treated with SEB/scFv-KJ16 lived longer than controls ($p < 0.05$) and mice treated with SEB and the folate conjugate of scFv-KJ16 lived longer than mice treated with the non-conjugate form of the antibody ($p < 0.05$).

evaluate if sustained or more extensive T-cell infiltration can lead to higher survival rates.

SV11 tumors arise because SV40 large T antigen interferes with p53 and pRB, and this etiology may be similar to some human brain tumors. SV40 has been isolated from pediatric choroid plexus tumors and ependymomas (Bergsagel *et al.,* 1992). SV40-induced tumors are immunogenic if transplanted into congeneic C57BL/6 mice (Mylin *et al.,* 1995), but SV11 mice are apparently immunologically tolerized to the tumors during development, as are other SV40 transgenic strains. We have transplanted the choroid plexus tumors to an S.C. location in SV11 mice, and the tumors were not rejected (data not shown). At this point, we have not determined whether either of the treatments that prolonged survival generated a tumor-specific T-cell response. It may be that the folate conjugation and targeting of T cells involves both redirected killing of tumor cells and the generation of tumor-specific T cells that have overcome tolerance, enhancing a process that may have occurred in animals treated with SEB and scFv-KJ16 without folate.

We have investigated the relationship of the blood-brain barrier and blood cerebrospinal fluid barrier to this treatment strategy. As the tumors develop, there are multiple foci in the lateral ventricles and 4th ventricle. The smaller tumors have an intact barrier (as revealed by the absence of murine IgG and exclusion of Evans blue/albumin), whereas the larger tumors have lost the barrier. Although there is heterogeneity in the density of the T-cell infiltrate in different foci, it is only partly accounted for by the blood-brain barrier. For example, there are definite small tumors with an apparently intact blood-brain barrier that also have infiltrating T cells. With adoptively transferred T cells incubated with an IgG form of anti-CD3 antibody/folate conjugate and infused directly into the brain, we have observed retention of T cells and a similar prolongation of survival (data not shown).

With systemic treatment, we observed few T cells associated with regions of normal choroid plexus, which also expresses the high-affinity folate receptor on its apical surface (Patrick *et al.,* 1997; Weitman *et al.,* 1992) and no infiltration of T cells into other normal tissues that have lower levels of the high-affinity folate receptor such as kidney and lung. Consistent with this finding, there have been no signs of obvious neurological problems in tumor-bearing or normal C57BL/6 mice treated with the bispecific agents described in this study, nor, apparently, in ovarian cancer clinical trials using conventional anti-FR bispecific antibodies (Mezzanzanica *et al.,* 1991). It may be that the blood cerebrospinal fluid barrier is intact in the choroid plexus, and the tumor vasculature is partially compromised even in small tumors. In this regard, there is a substantial body of literature supporting the notion that activated T cells can penetrate the blood-brain barrier, but penetration is enhanced through a partially disrupted barrier (Fabry *et al.,* 1995). This ability to penetrate the blood-brain barrier suggests that strategies to enhance T-cell activity in brain tumors may have advantages over strategies that rely on the penetration of antibodies alone.

The general approach of coupling a small high-affinity ligand to antibodies against immune effector cells (Chen *et al.,* 1995; Kranz *et al.,* 1995) should be widely applicable to any type of cancer in which a high-affinity ligand against a tumor-associated molecule can be identified. The folate/antibody approach may work more effectively against tumors such as ovarian carcinomas that do not have the complication of the blood-brain barrier and that may be more accessible by T cells. It is also likely that the ligand-targeting approach described here can be used to enhance other aspects of a T-cell response. In particular, folate conjugation to antibodies against T-cell co-stimulatory receptors such as CD28 may elicit enhanced T-cell activation at the site of the tumor, thereby combining activation and targeting while limiting systemic toxicity (Jung *et al.,* 1991).

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INTRACEREBRAL BISPECIFIC LIGAND-ANTIBODY CONJUGATE INCREASES SURVIVAL OF ANIMALS BEARING ENDOGENOUSLY ARISING BRAIN TUMORS.

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Immunotherapy of Brain Tumors

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Abstract

Bispecific antibodies capable of simultaneously binding a tumor surface antigen and the T cell receptor/CD3 complex are capable of inducing polyclonal immune effector cells to destroy targeted tumor cells. Bispecific antibody immunotherapies have shown some promise against tumors of hematopoietic origin such as lymphomas, but use of bispecific antibodies for the treatment of solid tumors has been less fully explored. To test the preclinical potential of bispecific antibody therapy against an endogenously-arising solid brain tumor, we have utilized a novel variation of conventional bispecific antibodies, referred to as bispecific ligand-antibody conjugates, to target choroid plexus tumors. The bispecific ligand-antibody conjugate described in this study is a chemical conjugate between an anti-CD3 monoclonal antibody and folic acid, the ligand for a high-affinity surface receptor expressed on the surface of choroid plexus tumors. SV11 mice transgenic for SV40 large T antigen and its promoter develop solid choroid plexus tumors in the brain. We demonstrate that choroid plexus tumor cells are susceptible *in vitro* to cytolysis mediated by cytotoxic T cells in the presence of the bispecific ligand-antibody conjugate in a folate competible-manner. Adoptive immunotherapy studies demonstrate the potential benefits of the bispecific ligand-antibody conjugate *in vivo*. The bispecific conjugate is capable of retaining adoptively transferred T lymphocytes specifically within tumor tissue for periods of up to at least a week. Further, following intracerebroventricular injection of bispecific conjugate and splenocytes containing activated cytotoxic T cells, T cells were observed to penetrate to interior regions of the tumor. A single treatment of adoptively delivered activated effectors and bispecific conjugate into the brain ventricles was insufficient to produce significant increases in survival of SV11 mice, but repeated treatment through indwelling cannulas prolonged survival of animals treated with activated effectors and bispecific ligand-antibody conjugate compared to animals treated with activated effectors or saline alone. This study demonstrates that the SV11 model may be useful for preclinical evaluation and optimization of bispecific ligand-antibody conjugate treatments of solid tumors.

Introduction

Tumors may evade immune recognition by a number of mechanisms. The lack of appropriate antigen presentation and/or costimulation by tumors is one means of preventing specific T lymphocyte recognition. In addition, the microenvironmment in which a tumor arises may actively suppress the initiation or execution of a competent specific immune response to tumor.

The brain has classically been considered an immune privileged site. The blood-brain barrier (BBB) is thought to prevent trafficking of unactivated lymphocytes through brain parenchyma, and the decreased surveillance may lower the probability of specific recognition of brain tumors. Molecular factors blocking the activation of immune effectors cells (e.g. transforming growth factor-β or prostaglandin E_2) are released by cells in the brain including microglia, astrocytes, and certain types of brain tumors (Fabry *et al.*, 1995). Systemic release of immunosuppressive factors by some brain tumors may further hamper the ability of the immune system to mount or maintain a response to tumor and, in some cases, may cause systemic immunosuppression (Van Meir, 1995). In light of these concerns, therapeutic strategies that overcome endogenous lymphocyte activation barriers may be useful.

One means of circumventing possible endogenous activation barriers is to activate effector lymphocytes *ex vivo*. Some clinical studies have focused on adoptive transfer of lymphokine activated killer (LAK) cells (Hayes *et al.*, 1995; Smith *et al.*, 1996). Some patients showed improvement when treated with LAK cells, but the recurrence of primary malignancy was high. This finding may be due to the polyclonal nature of the LAK cells that largely are not specific to the tumor under treatment. Adoptive transfer studies may benefit from approaches that redirect polyclonal effector cells to specifically lyse tumor cells.

A strategy which has been shown to redirect cytolytic action of polyclonal cytotoxic T cells (CTL) specifically against various tumors is bispecific antibodies. Simultaneously specific for a tumor surface antigen and an immune effector triggering molecule, bispecific antibodies can juxtapose CTL to tumor cells and mediate the specific redirection of polyclonal immune effectors

against tumor cells. Bispecific antibodies circumvent the requirement for tumor antigen presentation to CTL by directly signaling via immune effector surface molecules (e.g. T cell receptor/CD3 complex) responsible for stimulating cytotoxic action. Variations of the bispecific antibody strategy have been shown to be successful in numerous animal models and are currently being employed in clinical trials (e.g., Canevari *et al.*, 1995).

Whereas most animal studies employing bispecific antibodies have concentrated on hematopoietic tumors, solid tumors may be a more difficult challenge. Targeting and maintaining sufficient immunotherapeutic reagents or active immune effector cells in the interior of tumors may be an obstacle to bispecific antibody treatment of solid tumors. As with some other immunotherapeutic strategies (e.g. transfection of tumor cells with immune activating cytokines), reported success against solid tumors using bispecific antibody therapy is generally limited to preventing the growth of small tumor burdens transplanted into animal models rather than rejecting an established, solid tumor of endogenous origin.

One model for endogenously arising brain tumors is SV11 mice that are transgenic for large T antigen (Tag) and its associated promoter derived from the simian virus 40 (SV40) genome (Van Dyke *et al.*, 1987). SV11 mice develop choroid plexus tumors (CPT) with 100% penetrance and become moribund at approximately 100 days of age (Van Dyke *et al.*, 1987). The mechanism of tumorigenesis in SV11 mice involves direct inhibition of tumor suppressors p53 and pRB by Tag in choroid plexus epithelial cells. The reason for the selective expression of Tag in the choroid plexus epithelium is unknown. SV40 Tag sequences have been found in a majority of pediatric ependymomas and half of the pediatric choroid plexus tumors tested (Bergsagel *et al.*, 1992). In fact, intact virions of SV40 have been isolated from some of these tumors (Lednicky *et al.*, 1995). Speculation of a viral etiology for pediatric ependymomas and CPTs suggests that CPTs which develop in SV11 mice may be analogous to the development of their human counterparts and hence useful for the study of various immunotherapeutic regimes aimed at eliminating solid tumors.

Previously, we described a novel bispecific antibody targeting strategy where the small ligand folic acid was chemically coupled to anti-T cell receptor (TCR) antibodies (Kranz *et al.*,

1995). These bispecific ligand-antibody conjugates demonstrated specific and sensitive ability to redirect CTL to lyse tumor cells expressing the high affinity folate receptor (FR) *in vitro*. FR has been suggested as a potential tumor associated antigen useful for immunotherapeutic targeting of certain human neoplasms (Bolhuis *et al.*, 1992). Among neoplasms expressing FR are CPT, ependymomas, >95% of ovarian carcinomas, and ~30% of mammary adenocarcinomas (Buist *et al.*, 1993; Coney *et al.*, 1991; Ross *et al.*, 1994) . SV11 choroid plexus tumors express FR and thus may be useful for testing and optimizing the bispecific ligand-antibody conjugate strategy *in vivo* (Patrick *et al.*, 1997). We have recently shown that treatment of SV11 mice with a single-chain Fv antibody against Vβ8 T cell receptors conjugated with folate caused T cell infiltration of the tumors and significantly prolonged survival (Roy *et al.*, 1998). In the present study we used an anti-CD3 antibody conjugated to folic acid that targets all T cells, and delivered the bispecific ligand-antibody conjugate directly into the brain.

We first determined whether an anti-CD3 antibody/folate conjugate is capable of redirecting CTL against CPT cells. The anti-murine CD3 antibody 2C11 conjugated to folate was capable of redirecting both monoclonal and polyclonal CTL to specifically lyse CPT cells *in vitro*. Adoptive transfer of polyclonal CTL and 2C11/folate conjugate directly in the lateral ventricles of the brain of SV11 animals lead to retention of T lymphocytes in CPT within the interior of the tumor. In contrast, rapid clearance of CTL from the brain was observed in the absence of bispecific ligand-antibody conjugate. Finally, continued adoptive therapy of SV11 mice with CTL and bispecific ligand-antibody conjugate conferred a significant improvement in survival compared to animals treated with CTL or saline alone.

Methods

Mice. C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in animal facilities at the University of Illinois. The SV11 transgenic line was obtained from Terry Van Dyke (University of North Carolina). SV11 males heterozygous for Tag were mated to

C57BL/6 females. Progeny were screened by PCR for the presence of Tag (Patrick *et al.*, 1997). Prior to use in adoptive transfer treatment regimes mice were placed on low folate chow for a period of at least one week to reduce the level of serum folates that could potentially compete with the bispecific ligand-antibody conjugate for binding to FRs on CPT. All studies described were approved by the Laboratory Animal Care Advisory Committee and conducted in accordance with NIH guidelines for the care and handling of laboratory animals in experimental studies.

Cell lines and antibodies. All cell lines were incubated in a humidified incubator at 37° C and 5% CO_2. F2-MTX^{r}A, a non-adherent DBA/2-derived erythroleukemia line (Brigle *et al.*, 1991), was maintained in RPMI 1640 medium containing 5 mM HEPES, 10% (vol/vol) heat-inactivated fetal bovine serum, 1.3 mM L-glutamine, 50 mM 2-mercaptoethanol, 100 units/ml penicillin, and 100 units/ml streptomycin. Cytotoxic T lymphocyte clone 2C, a mouse alloreactive cell line specific for L^d, was maintained in the same RPMI medium described above and supplemented with 10% (vol/vol) supernatant from concanavalin A-stimulated rat spleen cells, 5% α-methylmannoside, and mitomycin C-treated BALB/c spleen cells (L^d) as stimulators (Kranz *et al.*, 1984). Hybridoma 2C11 (Leo *et al.*, 1987), a hamster IgG specific for the murine CD3ε subunit, was purified from ascites by ammonium sulfate precipitation and Protein A-Sepharose. Hybridoma 37.51 (Gross *et al.*, 1992), a hamster IgG-secreting line specific for murine CD28, was cultured in serum-free RPMI 1640 medium and purified by passage over a Protein-G Sepharose column. Rabbit antiserum to murine FR (kindly provided by Dr. Kevin Brigle) and normal rabbit antiserum (kindly provided by Steve Miklasz) was utilized in flow cytometry without further processing.

Flow cytometry. CPT cells were mechanically dissociated through wire mesh and separated by sedimentation into individual cells and small aggregates. Red blood cells were lysed by incubation in lysing buffer (0.14M NH_4Cl, 0.017M Tris, pH 7.2) for 5 minutes at 37° C. Isolated CPT cells were incubated with polyclonal rabbit antiserum to murine FR followed by fluorescent goat anti-

rabbit secondary (Kirkegaard & Perry Laboratories, Gaithersburg, MD) or secondary antibody alone. Cells were analyzed on a Modified Coulter EPICS 753 flow cytometer (Coulter Corp., Miami, FL) with Cyclops version 3.14 software for the percentage of CPT cells expressing FR. Splenocytes were labeled with a combination of fluorescent anti-CD4, CD8, and CD69 primary antibodies (Pharmingen, San Diego, CA). Cell were analyzed on a Coulter XL-MCL flow cytometer with Coulter System II software. Dead cells were excluded on the basis of high- or low-angle light scatter.

Preparation of antibody/folate conjugate. Conjugation of folic acid to purified 2C11 was performed as described previously (Kranz *et al.*, 1995). Briefly, folic acid (Sigma, St. Louis, MO) was dissolved in DMSO to a final concentration of 6.7 mM. EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, Pierce, Rockford. IL) was added to a final concentration of 33.5 mM (1:5 folate/EDC ratio) and incubated for 30 minutes at room temperature in the dark. Reacted folate/EDC was added to 2C11 antibody (1 mg/ml) in 0.1M MOPS pH 7.5 at a 100:1 folate/antibody ratio. Further incubation for one hour at room temperature in the dark was followed by passage over a Sephadex G-25 (Sigma, St. Louis, MO) column equilibrated in phosphate-buffered saline. The excluded-peak fractions containing conjugated antibody were pooled and analyzed spectrophotometrically for determination of folate density. Densities averaged 4-8 folates/antibody. 2C11/folate bispecific ligand-antibody conjugates were stored at $4^{o}C$ in the dark.

Cytotoxicity assays. Mechanically-dissociated choroid plexus tumor cells or F2-MTX^{r}A cells were labeled with 50 µl of ^{51}Cr (2.5 mCi/ml) for 1 hour at $37^{o}C$, washed repeatedly in folate-free RPMI 1640 containing 5% (vol/vol) fetal bovine serum, and subsequently used in 96-well plate cytotoxicity assays at $2x10^{4}$ cells per well. 2C11/folate conjugate was added to triplicate wells at 150 ng/ml. For folate-inhibition studies, free folate was added at various final concentrations ($2.5 \times 10^{-7} - 2.5 \times 10^{-11}$ M). Activation of CTL clone 2C effector cells was as described above and

added at an effector to target ratio of 10:1. Activation of splenocytes from C57BL/6 or SV11 mice was induced by an intravenous injection of 10 μg 2C11 antibody. The spleen was removed from these mice 48 hrs later (optimal time for CTL activation, unpublished observation, T.A.P.). Splenocytes were isolated by disruption through wire mesh, and red blood cells lysed by incubation in lysing buffer for 5 min. at 37°C. Remaining splenocytes were washed three times in folate-free RPMI 1640 medium containing 5% (vol/vol) heat-inactivated fetal bovine serum and added at effectors to target ratios of 100:1, 31:1, 10:1, 3:1, or 1:1 as indicated. Plates were incubated at 37°C for four hrs in a humidified, 5% CO_2 incubator. Culture supernatants were removed for γ counting. Specific ^{51}Cr release was calculated by standard methods [% specific ^{51}Cr release = (experimental counts-spontaneous counts)/(maximal counts-spontaneous counts) x 100].

***In vivo* activation of splenocytes for adoptive transfer.** C57BL/6 mice were injected intravenously with 10 μg anti-CD3 antibody 2C11 and harvested as described under cytotoxicity assays. Splenocytes were washed with phosphate-buffered saline rather than folate-free RPMI 1640. In cases were splenocytes and bispecific ligand-antibody conjugate were simultaneously transferred, splenocytes and the conjugate were incubated on ice for at least 30 minutes.

T lymphocyte retention in CPT *in vivo*. SV11 mice aged 90-100 days and having been placed on low folate chow for a period of not less than a week were anesthetized with 1.7 mg/20 g body weight ketamine and 250 μg/20 g body weight xylazine in sterile phosphate-buffered saline injected intraperitoneally. Mice scalps were shaved and pre-scrubbed with betadine. Surgery was performed using aseptic techniques. An incision of approximately 1 cm was made along the mid-sagittal plane of the scalp. A stereotaxic device was employed to determine coordinates 1 mm caudal and 1 mm lateral of bregma. The skull was perforated with a drill. Intracerebroventricular (ICV) injections (either, saline alone, 2 x 10^6 activated splenocytes, or 2 x 10^6 activated splenocytes and 100 ng 2C11/folate conjugate) were delivered 2.5 mm deep of dura (location of

superior horn of lateral ventricle) in a total volume of 5-10 µl at a rate of 2 µl/min, using a Hamilton syringe with an attached 28 gauge needle. Following a five-minute latency post-delivery, the syringe was removed and the skull sealed with bone wax. The scalp was sutured or sealed with Vetbond (3M, Minneapolis, MN). Animals were anesthetized with ketamine/xylazine at 24 hrs following ICV delivery. Animals were perfused with AZF fixative (Newcomer Supply, Middleton, WI) at room temperature. Brain, spleen, and thymus were removed from each animal and bathed in fixative overnight at room temperature.

Tissue preparation and histochemistry. AZF-fixed brains were blocked into 5-6 transverse sections at designated points (mid-IVth ventricle, rostral cerebellum, and approximately every 2.5 mm further rostral) the day following fixation and paraffin embedded along with spleen and thymus samples from the same mouse. All brain sections, spleen and thymus to be analyzed by immunohistochemistry were cut and mounted as three micron sections on the same slide. Slides were deparaffinized in xylene, rehydrated to phosphate-buffered saline, blocked with Superblock (Pierce, Rockford, IL), and incubated overnight with rabbit anti-human CD3 (murine CD3 cross-reactivity; DAKO, Carpinteria, CA) at 4^{o}C. Primary antibody was followed by biotinylated goat anti-rabbit and subsequently by avidin-biotin-HRP (Vector, Burlingame, CA). Nickel-cobalt-enhanced diaminobenzidine (DAB, Pierce, Rockford, IL) chromagen was used for detection. Slides were counterstained in methyl green, dehydrated, and coverslipped with Permount. Sections of spleen and thymus served as both positive and negative controls with respect to regions labeling positive for the presence of CD3. Analysis of tumor area was determined using NIH Image at 25x magnification. Individual T cells associated with tumor were counted at 100x magnification for density calculations (T cells/unit area).

Apoptosis tissue stains.

Tissue was prepared as above. Adjacent tissue sections were placed on separate slides for comparison of T lymphocytes presence and apoptosis. A TUNEL assay kit (Trevigen,

Gaithersburg, MD) was utilized to detect apoptosis. Briefly, tissue was deparaffinized in xylene and an ethanol series followed by several washes in distilled water. Proteinase K (20 μg/ml) was added to each slide, plastic coverslipped, and incubated at room temperature for 15 minutes. Slides were immersed in 2% H_2O_2 in methanol for five minutes before transferring slides to Labeling buffer. Manufacturer's labeling reaction mixture was added to each slide, plastic coverslipped, and incubated in a 37°C humid chamber for 1 hour. Following labeling reaction, slides were placed in stop buffer for five minutes, washed three times with PBS over a five minute period, and incubated with streptavidin-HRP in PBS for ten minutes. Slides were washed again in PBS before addition of metal enhanced DAB chromagen. Following a five min incubation, slides were rinsed repeatedly in distilled water and subsequently counterstained with methyl green. Brain parenchyma, thymus, and spleen from experimental animals served as apoptosis controls as well as control slides provided by the manufacturer.

Timecourse of T lymphocyte retention in CPT *in vivo*. The protocol for adoptive transfer of splenocytes and 2C11/folate conjugate was repeated exactly as for T lymphocyte retention at 24 hrs, but animals were killed at various time points following ICV delivery ranging from 24 hrs to 7 days. Four animals receiving activated polyclonal splenocytes and 2C11/folate conjugate were killed at 24 hrs, one animal receiving activated polyclonal splenocytes and 2C11/folate conjugate was killed at each successive time point, 3 animals receiving activated polyclonal effectors alone were killed at 24 hrs, and 3 untreated animals were killed as a time zero control for background density of T cells normally found within CPT. Cross-sectional areas of lateral ventricle CPT from at least four separate regions of cortex were calculated on NIH Image, and the number of T cells within those areas quantified to yield T cell density within tumor.

Treatment of SV11 mice with a single adoptive transfer of activated splenocytes. SV11 mice aged 85-90 days, on low folate chow for at least one week, were injected ICV as described above. Mice were distributed to treatment groups randomly with the proportions of each

group matched for sex where possible. Mice received either an average of 3.5 x 10^6 activated splenocytes and 100 ng 2C11/folate (n=11), 3.5 x 10^6 activated splenocytes (n=7) or phosphate-buffered saline alone (n=11). In instances where 2C11/folate were added to effectors for ICV delivery, incubation was at least 30 minutes on ice prior to ICV delivery. Mice were monitored daily until one of three criteria establishing morbidity were met, at which time the mouse was killed, perfused, and brain, spleen, and thymus prepared for immunohistochemistry as described above.

Clinical evaluation of mice. Animals were weighed daily and monitored for level of activity and vestibular integrity. Loss of greater than 25% of pre-treatment body weight, lack of responsiveness to external stimuli, or carrying head at an improper angle or inability to right self were established as sufficient criterion for morbidity classification.

Statistical methods. T cell densities were compared using the Students t-test. Survival analyses were based on age at first sign of morbidity according to the above criteria. Ages were entered into a standard Kaplan-Meier survival plot. Statistical p-values were calculated using the log-rank method on JMP software (SAS Institute, Inc., Cary, NC). Mean and median ages at morbidity are reported.

Comparison of *in vivo* and *in vitro*-activated splenocytes. Unactivated splenocytes were harvested from C57BL/6 mice and depleted of red blood cells with lysing buffer as described above. Splenocytes were washed in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal bovine serum. Splenocytes were placed in 24-well tissue culture plates at 1.5x10^6 cells per well with 100 ng/ml anti-CD3 antibody 2C11 and 5 μg/ml - 8 ng/ml anti-CD28 antibody 37.51 or as indicated. Splenocytes were incubated for 72 hrs at 37^{o}C in a humidified, 5% CO_2 incubator. All cell preparations were >95% viable. Splenocytes activated *in vivo* as described above were compared to splenocytes activated *in vitro* by flow cytometry for percent composition

of $CD8^+$ cells. Cytotoxic assays similar to that described above using the FR^+ cell line, F2-MTX^rA as tumor target were used to compare killing efficiencies of the two T lymphocyte activation methods. The ER_{50} (effector:target ratio giving half-maximal lysis) was calculated by linear regression of data points in the linear region of the curve and was used to compare the lytic potential of *in vivo* and *in vitro*-activated splenocytes.

Treatment of SV11 mice with multiple adoptive transfers of *in vitro*-activated splenocytes. Mice aged 65-70 days were prepared for surgery as described above. Permanent cannulas were placed bilaterally in the skull of SV11 mice. Knotted PE-50 tubing was inserted bilaterally 1.6 mm caudal and 0.6 mm lateral of bregma to a depth of 3 mm below the skull. Cannulas were held in place with dental cement anchored to a "00" machine screw placed approximately 5 mm rostral of the left cannula. In some cases, stylets fashioned from 28 gauge stainless steel tubing were used to prevent cannula occlusion. Animals were allowed a two week period to recover from surgery. Morbidity due to surgery was minimal (<5%). At approximately 85 days of age, mice were injected bilaterally (when possible) with 5×10^5-2.5×10^6 splenocytes and 100 ng 2C11/folate conjugate (n=18), 5×10^5-2.5×10^6 splenocytes (n=11), or saline (n=6) in a total volume of 10 ml. Splenocytes were activated *in vitro* (as described above) with 100 ng/ml 2C11 and 0.5-1 μg 37.51. Splenocytes were harvested and washed three times in phosphate-buffered saline prior to ICV injection.

Results

FR expression by CPT cells.

The first objective was determining whether CTL could be redirected by the 2C11/folate conjugate to lyse cells from a solid choroid plexus tumor. To establish the proportion of CPT cells that are positive for FR expression and may serve as potential targets for the bispecific ligand-antibody conjugate targeting strategy, a preparation of dissociated choroid plexus tumor cells from an SV11 mouse was labeled with a polyclonal serum to murine high-affinity folate receptor (Fig.

1). Flow cytometric analysis indicated that a large fraction (>97%) of the isolated tumor cells express detectable FR.

Susceptibility of CPT cells to a monoclonal CTL *in vitro*.

To test the susceptibility of these CPT cells to CTL-mediated lysis, dissociated CPT cells were incubated with the CTL clone 2C and 2C11/folate conjugate (150 ng/ml) in a standard ^{51}Cr release assay. Various concentrations of free folate were added to establish if redirection of cytotoxic activity is specifically mediated through FR expressed by the CPT cells. As shown in figure 2, CTL clone 2C is capable of lysing choroid plexus tumor cells in a folate-dependent manner. CPT cell incubation with CTL clone 2C in the absence of 2C11/folate conjugate resulted in no detectable lysis (data not shown) further suggesting that the 2C11/folate conjugate is responsible for the redirection of 2C toward FR-expressing cells.

Susceptibility of CPT cells to polyclonal CTL *in vitro*.

Having established that CPT cells were susceptible to CTL clone 2C, we next determined whether activated splenocytes containing a activated population of polyclonal CTLs derived from C57BL/6 or SV11 mice could be redirected to lyse CPT cells *in vitro*. C57BL/6 or SV11 mice were injected intravenously with 10 μg of 2C11 antibody, which produces an active splenocyte subpopulation of CTL capable of cytolysis 24-48 hrs later as measured by standard ^{51}Cr release assays (unpublished results, T.A.P.). Splenocytes were harvested from these animals 48 hrs after 2C11 administration and utilized in a standard ^{51}Cr release assay against CPT cells. Figure 3A demonstrates that in the absence of 2C11/folate conjugate, both activated and unactivated populations of splenocytes have negligible lytic activity toward choroid plexus tumor cells in a four-hr assay. Figure 3B demonstrates that the presence of 2C11/folate conjugate and activated CTL from either C57BL/6 or SV11 mice can be redirected to lyse CPT cells, while CTL not pre-activated are incapable of inducing lysis in a four-hr assay. These data indicate that CPT cells are susceptible to lysis by activated polyclonal CTL from either SV11 or C57BL/6 mice and that

2C11/folate conjugate is a capable mediator of redirecting the lytic potential of these CTL. Similar to the data presented for monoclonal CTL 2C, the redirection of lytic activity by these *in vivo*-activated CTL was competable by addition of free folate (data not shown).

2C11/folate conjugate retains T cells within choroid plexus tumors *in vivo*.

To assess the action of the bispecific ligand-antibody conjugate at juxtaposing activated polyclonal CTL against CPT *in vivo*, we utilized preparations of activated C57BL/6 splenocytes described above incubated with 2C11/folate conjugate or vehicle. An average of 2×10^6 cells suspended in PBS was adoptively transferred directly into the superior lateral ventricles in a volume of 5-10 µl. Animals were killed 24 hrs following ICV delivery. Splenocytes injected in the absence of 2C11/folate conjugate showed few T cells associating with choroid plexus tumor after 24 hrs (Fig. 4B). In contrast, when splenocytes were injected with 2C11/folate conjugate, a significant increase in the density of T cells was present throughout CPT 24 hrs later ($p < 0.01$ vs. splenocytes without 2C11/folate conjugate) (Fig. 4A). Many of the T cells associating with CPT appear to have penetrated to interior depths of the solid tumor. Further, in both 2C11/folate conjugate treated and untreated animals, few T cells are found in normal brain parenchyma at 24 hrs and those observed were typically clustered along myelin tracts or adjacent to vessels suggesting an outward emigration.

Adjacent tissue sections were analyzed separately for the presence of $CD3^+$ cells or apoptosis twenty-four hrs after ICV administration of activated splenocytes and 2C11/folate conjugate (Fig. 4C-D). Apoptotic cells were clustered variously throughout tumor tissue. Juxtaposed to many of the apoptotic cells are $CD3^+$ cells, but the apoptotic cells do not simultaneously stain for the CD3 antigen. Apoptosis was not detected among normal choroid plexus epithelium, brain parenchyma, or the ependymal lining of the brain ventricles (data not shown). Marginal apoptosis of CPT cells was observed in animals treated with activated splenocytes without 2C11/folate conjugate or saline alone (Fig. 4E-F). Further, apoptosis was not

observed in CPT of animals treated with activated splenocytes and 2C11/folate conjugate forty-eight hrs or longer after ICV transfer (data not shown).

2C11/folate conjugate retains T lymphocytes in CPT for periods of up to a week.

A timecourse study revealed the duration the 2C11/folate conjugate retained T cells in tumor tissue. SV11 mice were injected ICV with *in vivo*-activated splenocytes in the presence or absence of 2C11/folate conjugate. Subsequently, animals were perfused at various time points and brain sections stained for the presence of CD3. Figure 5 illustrates the mean density of T cells in 2C11/folate conjugate-treated animals over time compared to animals receiving T cells without 2C11/folate conjugate or to untreated SV11 animals. Corroborating results shown in figure 4B, at 24 hrs post-ICV delivery the density of T cells in CPT tissue of animals receiving no 2C11/folate conjugate at 24 hrs was low with an average of less than 25 T cells/mm^2. Similarly, the baseline presence of T cells within untreated SV11 animals was low, averaging 3 T cells/mm^2. In marked contrast, at 24 hrs post-ICV delivery animals receiving T cells with 2C11/folate conjugate had elevated levels of T cells among tumor tissue averaging over 600 T cells/mm^2 ($p<0.01$ presence vs. absence of 2C11/folate conjugate) and in certain regions T cell densities were over 1500 T cells/mm^2. Further, elevated numbers of T cells can be found in tumor tissue for up to seven days compared to the no 2C11/folate conjugate 24 hr time point. The density of T cells in CPT 24 hrs following delivery of activated splenocytes and unlabeled 2C11 antibody was similar to the density of delivering activated splenocytes alone (data not shown)

To control for the possibility that the T cells observed in the sections were not those adoptively transferred but immigrating endogenous cells, we analyzed the effects of injecting 2C11/folate conjugate or unlabeled 2C11 monoclonal antibody alone into the lateral ventricles of SV11 animals. Injection of 2C11/folate conjugate ICV without splenocytes results in the immigration of a negligible density of T cells into CPT (data not shown). Even under circumstances where a pre-activating dose of anti-CD3 antibody was administered systemically to SV11 animals 24 hrs prior to ICV delivery of 2C11/folate conjugate in attempts to promote CTL

activation and surveillance, a negligible increase in density of T cells was observed infiltrating into CPT. Collectively, the observations indicate that the most probable origin of the T cells within sections of the CPT are those which are adoptively transferred.

Survival analysis of SV11 animals with a single adoptive transfer of activated splenocytes and 2C11/folate conjugate.

SV11 mice received a single ICV injection of *in vivo*-activated splenocytes and 2C11/folate conjugate, splenocytes alone, or phosphate-buffered saline. Survival results indicated that administration of splenocytes and 2C11/folate conjugate in a single bolus ICV did not confer a statistically significant therapeutic advantage over administering T cells alone ($p>0.05$) or sham controls ($p>0.05$). While animal longevity was not extended, fewer animals appear to become moribund at younger ages (85-100 days) when treated with T cells (with or without 2C11/folate conjugate) compared to sham controls.

Comparison of *in vivo*- vs. *in vitro*-activated splenocyte effectors.

Flow cytometric analysis of *in vivo*-activated splenocytes harvested for ICV delivery demonstrate that the effector population of activated $CD8^+$ cells accounted for only 4-9% of total splenocytes compared with an average of 15-20% in a resting spleen (Fig. 6A-B)]. CD69, an early marker indicative of T lymphocyte activation (Allison *et al.*, 1995), was present on approximately 70-80% of $CD4^+$ or $CD8^+$ cells in animals receiving 2C11, but on less than 2% of T lymphocytes in animals not receiving 2C11. Thus, injection of 3.5×10^6 splenocytes resulted in fewer than 3.5×10^5 $CD8^+$ cytotoxic T cells actually delivered and fewer still likely capable of cytolysis. Due to the low proportion of $CD8^+$ cells rendered with *in vivo* activation, splenocytes were activated *in vitro* with a combination of anti-CD3 (2C11) and anti-CD28 (37.51) antibodies to expand the proportion of CTL effectors in the overall cell population. Splenocytes were incubated with 100 ng/ml 2C11 antibody and various doses of 37.51 to determine the optimal level for producing active CTL effectors. Activation of splenic T cells with CD28 costimulation after 72 hrs

consistently expanded the $CD8^+$ population to 60-70% of total cells regardless of the 37.51 dose tested (approximately ten times the percentage of $CD8^+$ cells found among *in vivo*-activated splenocytes). Representative flow cytometric data for the *in vitro*-activated splenocytes for $CD8^+$ cells are shown in Fig. 6C. CD69 expression is not observed on the predominance of T lymphocytes following *in vitro* activation most likely as a result of the relatively transient expression of this marker.

We tested the relative effectiveness of *in vitro*- and *in vivo*-activated splenocytes at lysing a FR-expressing target mediated by 2C11/fol redirection. Similar to *in vivo*-activated CTL, *in vitro*-activated CTL demonstrate the capacity to lyse the FR-expressing cell line F2-MTX^rA in the presence of 2C11/folate conjugate (Fig. 7A-B). Comparison of the lytic potential of these two effector populations revealed that the ER_{50} (effector:target ratio giving half-maximal lysis) of effector to targets is approximately six to ten-fold lower for *in vitro*-activated effectors (ten times fewer *in vitro*-activated splenocytes compared to *in vivo*-activated splenocytes). Given that the percentage of $CD8^+$ cells among *in vitro*-activated splenocytes is approximately ten-fold greater than that among the *in vivo*-activated splenocytes, the resulting ten-fold difference in the ER_{50} likely is a function of CTL number and not differences in CTL cytotoxic potency.

Survival analysis of SV11 animals treated with multiple adoptive transfers of *in vitro*-activated polyclonal effectors and 2C11/folate conjugate through indwelling cannulas.

Two groups of mice receiving *in vitro* -activated cells every three or five days, either with 2C11/folate conjugate (n=18) or without (n=11), were compared to a group of animals receiving saline (n=6). Survival analysis demonstrated that co-treatment of CPT with activated splenocyte effector cells and 2C11/folate conjugate improved survival over administration of T cells alone (p=0.001) or saline (p=0.003) (Figure 8). Mean time to morbidity of animals treated with T cells alone was 98 days (SD 1, median 98) while animals receiving 2C11/folate conjugate survived an average of 107 days (SD 2, median 107) indicating that the presence of 2C11/folate conjugate

conferred a therapeutic advantage. Mean time to morbidity of saline controls was 99 days (SD 1, median 98).

Discussion

The aim of this study was to determine whether a bispecific ligand-antibody conjugate could produce a significant improvement in survival of animals with an endogenously-arising solid brain tumor, when delivered directly to the tumor. The first objective was to determine whether CPT cells would be susceptible to bispecific ligand-antibody conjugate-mediated cytolysis by polyclonal effector cells derived from SV11 mice or congeneic C57BL/6 mice. Some types of brain tumors secrete substances that inhibit the activation of T cells, e.g. TGF-β. The possibility exists that the CPT in the SV11 animal provides a locally immunosuppressive microenvironment. Unactivated polyclonal splenocytes from SV11 animals showed no detectable lytic activity against CPT, suggesting that either the SV11 animal is tolerant to the presence of tumor or that the tumor may be locally or systemically immunosuppressing the mice. However, anti-CD3-activated polyclonal effectors from SV11 or C57BL/6 mice could be induced to lyse CPT in the presence of 2C11/folate conjugate, demonstrating that if there is systemic immunosuppression, it can be overcome. Further, these same activated polyclonal effectors from SV11 mice did not lyse CPT in the absence of 2C11/folate conjugate, suggesting that SV11 mice do not harbor expanded anti-tumor clones among splenocytes. Folate competiton of CTL-mediated lysis demonstrated that the 2C11/folate conjugate was responsible for the cytotoxic interaction. In addition, incubating target cells with active CTL and unlabeled 2C11 antibody did not lead to cytolysis of target cell lines *in vitro* (data not shown).

When administered *in vivo*, the 2C11/folate conjugate was capable of retaining T lymphocytes within CPT tissue, whereas these same effectors quickly cleared from the brain in the absence of 2C11/folate conjugate. These data suggest that the 2C11/folate conjugate is capable of contributing to the prevention of the normal migratory tendency of activated T lymphocytes. The

data also indicate that the 2C11/folate conjugate is capable of retaining T lymphocytes in CPT tissue for periods of up to a week at higher than background densities.

The first treatment protocol involved adoptive delivery of a single injection of activated splenocytes and 2C11/folate conjugate. The animals were treated at 85-90 days of age, a point at which the CPTs typically have progressed to Grade III and IV neoplasms. The survival of SV11 animals treated in this fashion did not significantly differ from that of animals treated with activated splenocytes alone or saline injected animals. Considering the relative density of T cells adhering to CPTs in the timecourse analysis, calculations indicate that perhaps one T cell is present for every 4-10 CPT cells at 24-48 hrs post-ICV delivery. The low proportion of $CD8^+$ cells among *in vivo*-activated splenocytes and the relatively short duration of peak retention of T cell density suggested the need for enriching the proportion of $CD8^+$ CTL among the splenocyte pool as well as devising a means to deliver multiple injections of CTL and 2C11/folate conjugate.

Activating splenocytes *in vitro* greatly expanded the CTL effector population. Although CTL activated in this fashion are not more potent against CPT cells than the *in vivo*-activated counterparts, more CTL could be delivered per injection. Delivering multiple infusions of splenocytes and 2C11/folate conjugate through indwelling cannulas increased SV11 animal survival. Activating splenocytes *in vitro* results in an effector CTL population that is pre-coated with unlabeled 2C11 antibody (flow cytometric results, data not shown). Repeated delivery of splenocytes alone into the lateral brain ventricles in these experiments actually includes delivery of 2C11 antibody. Because splenocytes pre-coated with 2C11 did not confer a therapeutic advantage over animals treated with saline, the 2C11/folate conjugate was responsible for conferring the beneficial therapeutic effect.

To further characterize CTL action against CPT *in vivo* and possible side effects to choroid plexus epithelium or healthy brain parenchyma, we performed apoptotic assays of tissue sections adjacent to those stained for CD3. Untreated SV11 animals commonly have evidence of a slight amount of background apoptosis in CPT tissue. Apoptosis of CPT cells of SV11 animals treated with saline or activated splenocytes alone was not appreciably different from untreated SV11

animals. We observed a greater extent of apoptotic staining in CPT of SV11 animals receiving 2C11/folate conjugate and activated splenocytes. Adjacent slide sections stained for apoptosis or the CD3 antigen showed apoptotic cells to be juxtaposed to cells staining for CD3, suggesting that the 2C11/folate conjugate may be redirecting CTL action against CPT cells. Apoptosis of non-tumor tissue was not observed in any of the treatment group animals nor in normal C57 mice treated under similar conditions.

T cells delivered into the lateral ventricles with 2C11/folate conjugate were found to penetrate tumors located in all regions of the lateral or third ventricles, but IVth ventricle tumors were typically devoid of T cell presence, suggesting that T cells are not penetrating the cerebral aqueduct following ICV delivery. A number of SV11 animals receiving CTL and 2C11/folate conjugate had small or negligible tumor loads in the lateral ventricles at later survival times, compared to the typically large lateral ventricle tumors of animals not receiving 2C11/folate conjugate, and large IVth ventricle tumors.

A potential cause for concern regarding the ICV treatment regime is that normal choroid plexus epithelium expresses the high-affinity folate receptor as do CPTs. Normal choroid plexus has a polarized distribution of FR concentrated on the apical surface facing the ventricular lumen directly exposed to the injected T cells and 2C11/folate conjugate (Patrick *et al.*, 1997). In numerous cases T cells were found associated with normal choroid plexus, although predominately in the presence of 2C11/folate conjugate. While the choroid plexus was sometimes altered in morphology compared to that of untreated animals, we were unable to detect damage to normal choroid plexus in treated mice, either by gross morphology or presence of apoptotic cells. We do not know why epithelial cells appeared resistant to T cell mediated lysis. Similarly, C57 mice treated with activated T cells and 2C11/folate conjugate did not shows any signs of short-term (12-24 hrs) or long-term (up to one yr) toxicity to normal choroid plexus.

Given the transgenic nature of the CPT, we did not expect any of the treatment regimes to produce a complete cure. All cells of the choroid plexus epithelium may serve as potential origins of tumorigenesis. SV11 animals commonly have multiple CPT foci in the lateral and IVth

ventricles at morbidity. A more realistic goal in this model is comparing increases in mean time to morbidity rather than expecting long-periods of disease-free remission.

While treatment of SV11 animals with multiple injections of splenocytes and 2C11/folate conjugate improved animal survival, there is clearly room for improvement. An important issue to address is the length of time retained CTL remain active and capable of cytolysis within the CPT. While T cells were observed adhering to CPT for periods beyond a week post-ICV delivery, the activational status of these cells is unknown. Considering that primary T cell activation is a transient process for an individual T cell, CTL are unlikely to remain active for extended periods of time (Speiser *et al.*, 1997). In addition, activation of CTL with 2C11 leads to a down-regulation of CD3 that may have reduced the potential efficacy of the bispecific ligand-antibody conjugate strategy for treatment of CPTs. As a potential means of promoting greater longevity of CTL activation, the application of additional bispecific ligand-antibody conjugates targeting costimulatory molecules (e.g. folate/anti-CD28, folate/anti-LFA-1, etc.) may simultaneously enhance primary activation or permit reactivation of adoptively transferred cells. An additional strategy may be to deliver blocking anti-CTLA-4 monovalent antibody fragments to reduce possible CTL down-regulation (Allison *et al.*, 1995).

Targeting sufficient numbers of activated effectors to tumor has been a primary concern for most immunotherapeutic strategies. The high affinity of the folate receptor for folate and the apparent capability of the 2C11/folate conjugate to keep T cells retained in tumor for periods of up to a week suggests that attaching a high-affinity ligand to anti-effector cell antibodies may be an effective means of targeting and retaining effector cells in tumor regions. The incorporation of bispecific ligand-antibody conjugates selectively stimulating costimulatory molecules on effector cells may promote sustained reactions to tumor, and perhaps the recruitment of endogenous effectors.

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Figure Legends

Fig. 1. Flow cytometric analysis of high-affinity folate receptor expression. Mechanically dissociated 3V11 choroid plexus tumor cells were labeled in the presence of normal rabbit anti-serum (A) or a rabbit polyclonal serum to murine folate receptor (B). Each were followed by an immunofluorescent goat anti-rabbit secondary.

Fig. 2. Specific redirection of CTL clone 2C cytotoxic activity against choroid plexus tumor cells by 2C11/folate conjugate. To test the susceptibility of choroid plexus tumor cells to lysis by CTL mediated through redirection by the 2C11/folate conjugate, ^{51}Cr-labeled choroid plexus tumor cells were incubated over a four hour period with CTL 2C, 150 ng/ml 2C11/folate conjugate, and

various concentrations of competing free folate. Results presented are the mean of triplicate samples.

Fig. 3. Specific redirection of activated splenocytes to lyse ^{51}Cr-labeled choroid plexus tumor cells. The capacity for *in vivo*-activated effector CTL among activated splenocytes to be redirected to lyse CPT cells by 2C11/folate conjugate was tested. Activated C57BL/6 (■), SV11 (◆), or unactivated SV11 (▲) splenocytes were incubated at various effector-to-target ratios for four hrs in the absence (A) or presence (B) of 2C11/folate conjugate and choroid plexus tumor cells. Specific ^{51}Cr release mediated by 2C11/folate conjugate redirection of CTL activity was competable by free folate (data not shown).

Fig. 4. Retention of T cells in CPT and apoptosis. Animals were infused ICV with activated splenocytes in the presence (A) or absence (B) of 2C11/folate conjugate and killed 24 hrs later. Tissue sections from multiple brain regions were stained for CD3. T cells are present throughout CPT tissue when 2C11/folate conjugate was administered (A), but T cells have migrated out by 24 hrs if 2C11/folate conjugate was not administered (B). Relatively few T cells were observed in normal brain parenchyma. Adjacent sections from an animal treated with 2C11/folate and activated splenocytes killed 24 hrs after adoptive transfer ICV were stained for CD3 (C) and apoptosis (D). Again, 2C11/folate conjugate retained T cells specifically within CPT (C). Cells staining for CD3 (C) are juxtaposed to apoptotic cells (D). Apoptosis was not observed in normal brain parenchyma or choroid plexus. Animals not receiving 2C11/folate conjugate revealed few retained T cells in CPT at 24 hrs (E) or apoptosis of tumor tissue on adjacent sections (F).

Fig. 5. T cell retention in choroid plexus tumor following intracerebroventricular delivery. Approximately 2 x 10^6 activated splenocytes were injected ICV in the presence (●) or absence (■) of 2C11/folate conjugate and compared to T cell density in CPT of untreated SV11 animals (▲). SV11 animals were killed at various time points following injection, perfusion fixed, and brains

mounted for anti-CD3 immunohistochemistry. Total tumor area of lateral ventricle CPT of either treated or sham control animals was calculated from 3-5 brain sections of differing regions and the T cells quantified associated with tumor. Vertical bars indicate SD. The number of animals used at each time point was: 0 hrs., n=3, 24 hrs., n=4 for each; 48 hrs., n=1; 72 hrs., n=1; and 168 hrs., n=1. Activated splenocytes with 2C11/folate differed from both untreated controls and activated splenocytes without conjugate, $p < 0.01$.

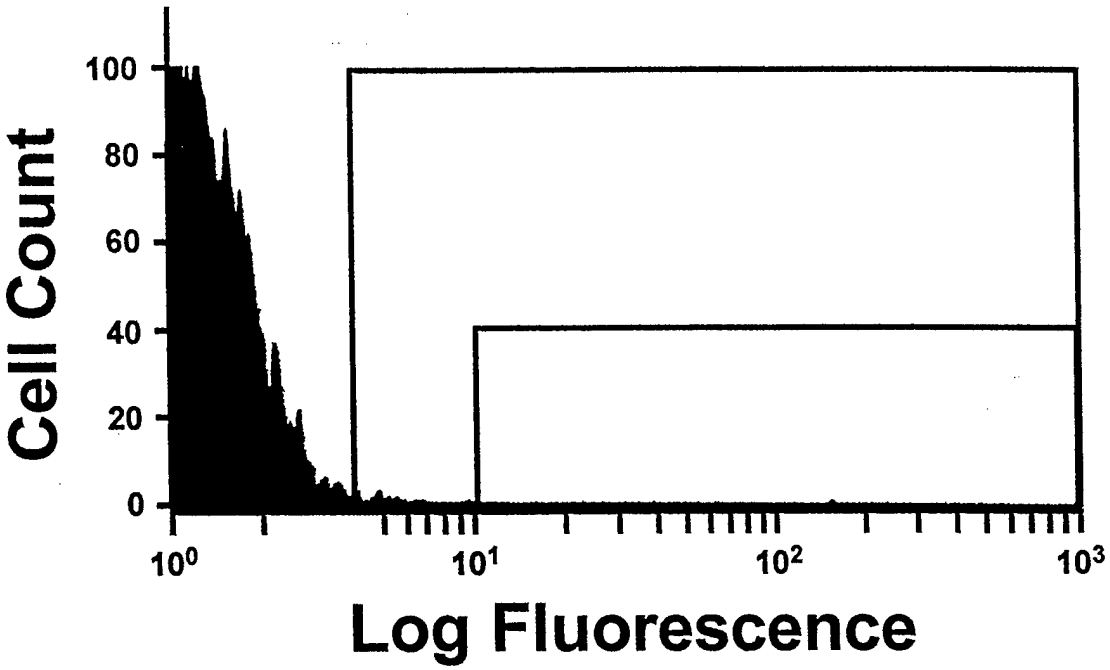
Fig. 6. Flow cytometric analysis of activated splenocytes. Resting (A) or *in vivo*-activated (B) splenocytes were labeled with a combination of labeled anti-CD4, CD8, and CD69 antibodies. Resting splenocytes have $CD4^+$ and $CD8^+$ populations of approximately 25-35% and 15-20%, respectively and low expression of the early activation marker CD69. Twenty-four hrs following a 10 μg injection of 2C11, the majority of $CD4^+$ and $CD8^+$ cells are positive for CD69 indicating an activated state, although the percentage of $CD8^+$ cells has dramatically decreased among splenocytes (< 8%). Splenocytes activated *in vitro* (C) were similarly labeled with a combination of anti-CD4, CD8 and CD69 antibodies following a 72 hr. incubation period with 100 ng/ml 2C11 and 0.5 mg/ml 37.51. The percentage of $CD8^+$ cells has expanded (68%). The early activation marker CD69 largely is not present on these cells indicative of marker turnover rather than an unactivated state.

Fig. 7. Comparison of *in vivo*- and *in vitro*-activated splenocyte retargeting. The FR^+ tumor target line F2-MTX^{r}A and various E:T ratios of each effector population were incubated with 150 ng/ml 2C11/folate. *In vivo*-activated effectors (A) were tested either in the presence (▲) or absence (◆) of 2C11/folate conjugate at E:T ratios of 100:1, 30:1, 10:1, and 3:1. Splenocytes activated *in vitro* (B) were stimulated with 100 ng/ml 2C11 and various concentration of 37.51: 5 μg/ml (◆), 1 μg/ml (■), 0.2 μg/ml (▲), 0.04 μg/ml (●), 0.008 μg/ml (✳). Splenocytes were also incubated with 100 ng/ml 2C11 alone (✚), 5 μg/ml 37.51 alone (✖), or media alone (▬). E:T ratios tested for all *in vitro*-activated splenocytes were: 30:1, 10:1, 3:1, and 1:1. Half-maximal

lysis (ER_{50}) was achieved at an E:T of approximately 30:1 by *in vivo*-activated splenocytes, but an ER_{50} of less than 5:1 for *in vitro*-activated splenocytes.

Fig. 8. Survival of cannulated SV11 mice treated intracerebroventricularly with multiple injections of in vivo-activated splenocytes and 2C11/folate conjugate. SV11 animals were injected ICV at approximately 85 days of age and every 3-5 days thereafter until morbidity. Animals received saline (- - -), n=6, pre-activated splenocytes (····), n=11, or pre-activated splenocytes and 1 μg 2C11/folate conjugate (——), n=18. Animals were assessed daily for alteration in body weight and vestibular complications. A 25% decrease in pre-treatment body weight or inability to right self served as criterion for morbidity. Animals treated with 2C11/folate conjugate and activated splenocytes lived significantly longer than animals treated with saline ($p = 0.003$) or activated splenocytes without conjugate ($p = 0.001$).

A.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40
20
0
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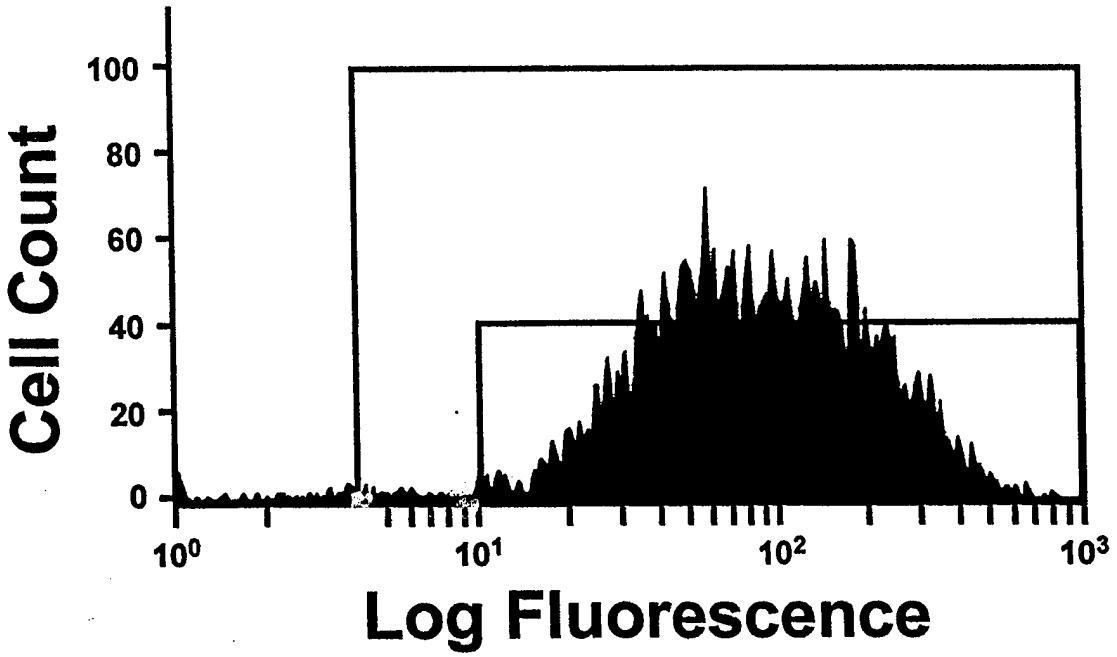
10^1
10^2
10^3
Log Fluorescence

B.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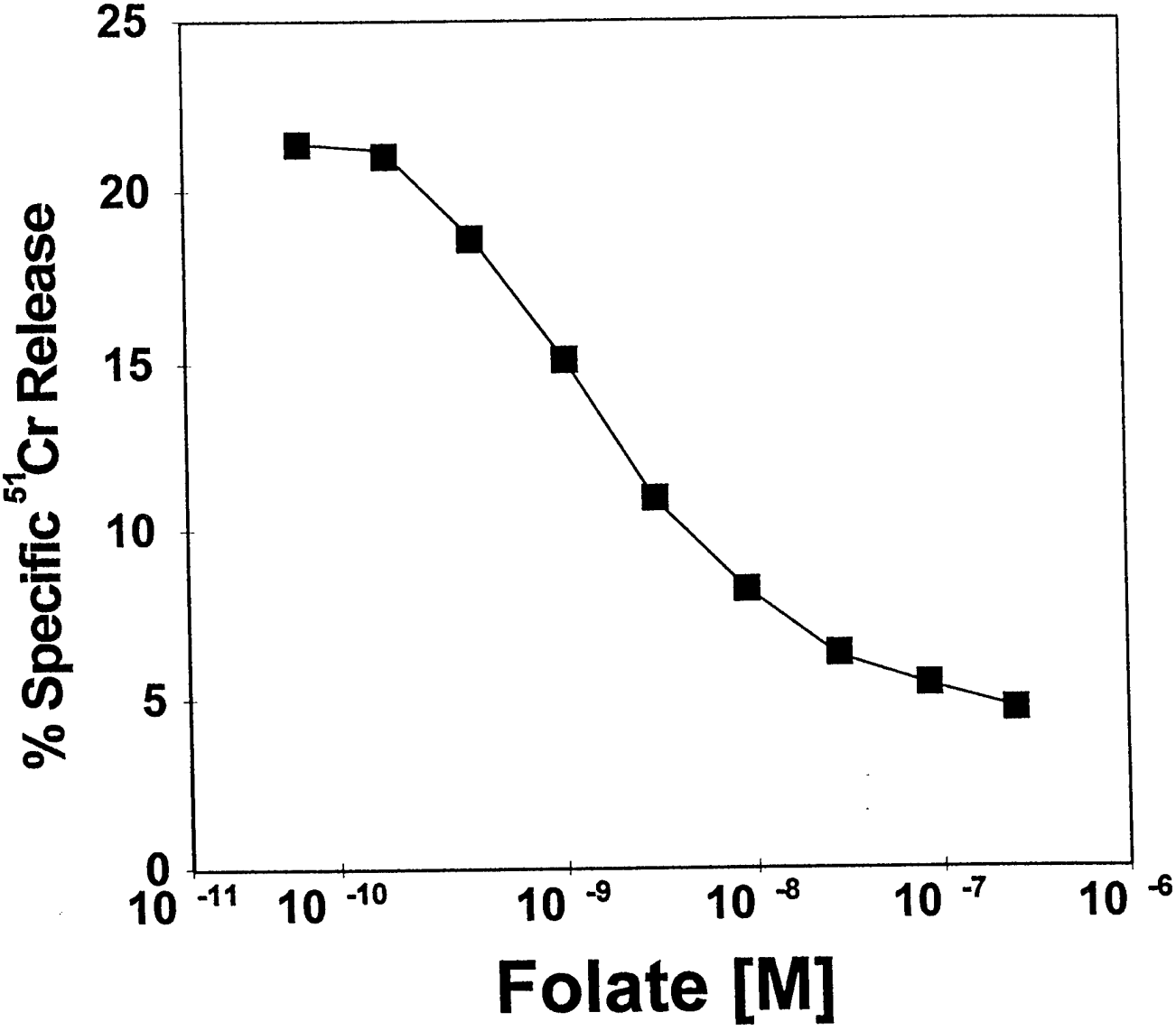
25
20
15
10
5
0
% Specific 51Cr Release
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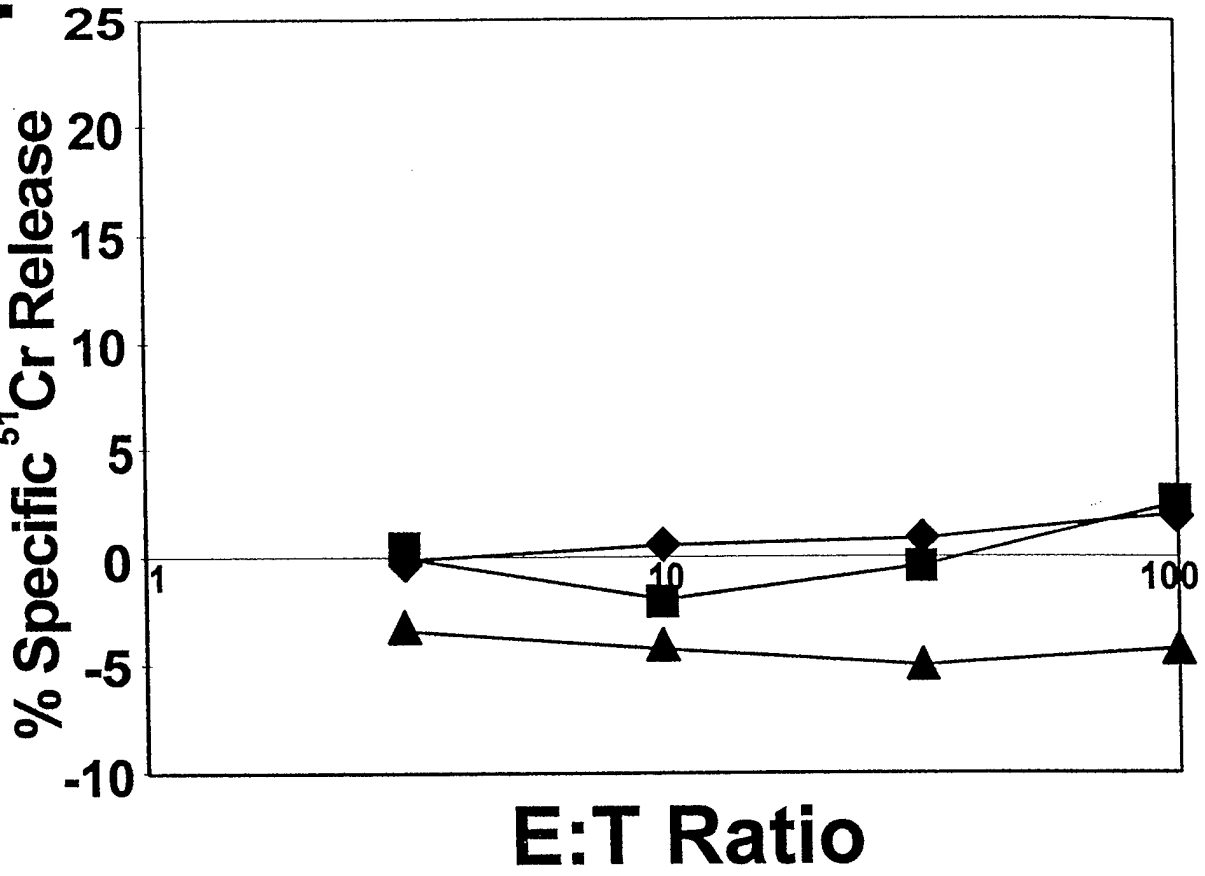
10 -10
10 -9
10 -8
10 -7
10 -6
Folate [M]

A.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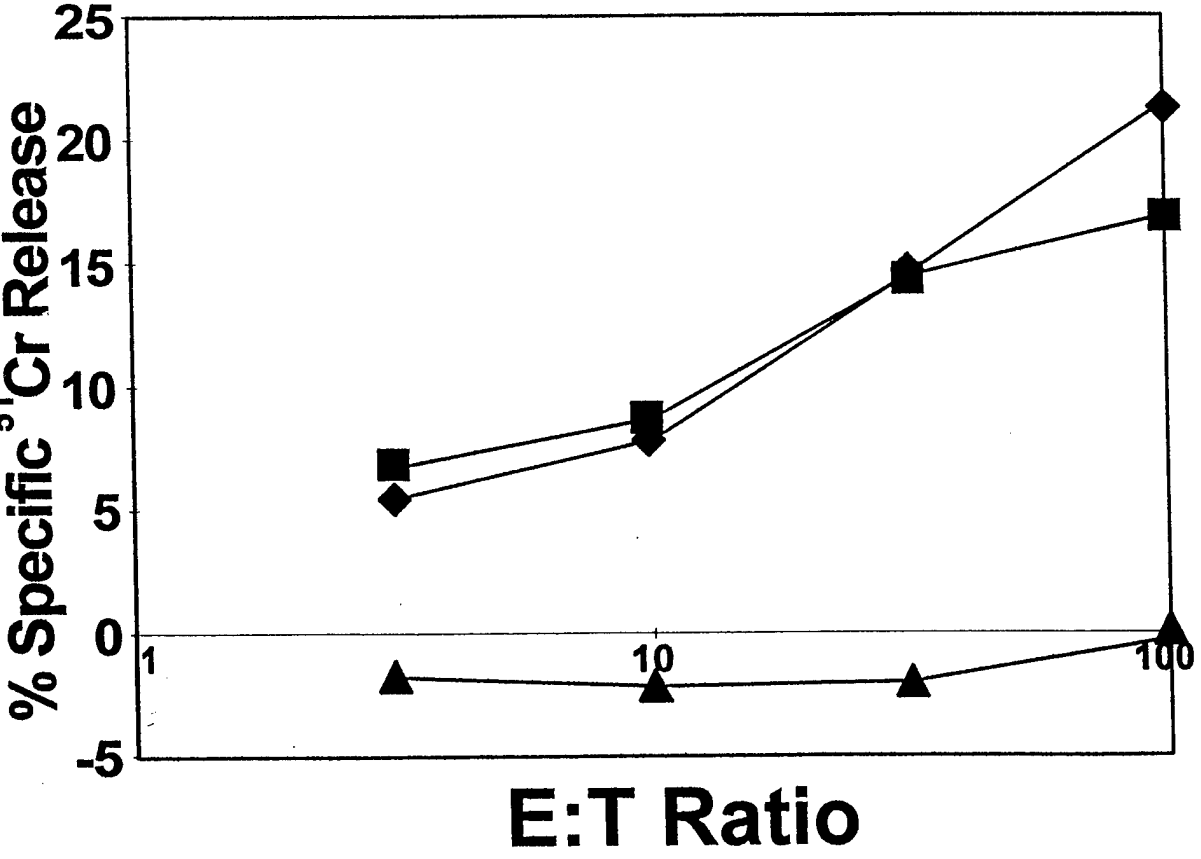
20
15
10
5
0
-5
-10
% Specific 51Cr Release
1
10
100
E:T Ratio

B.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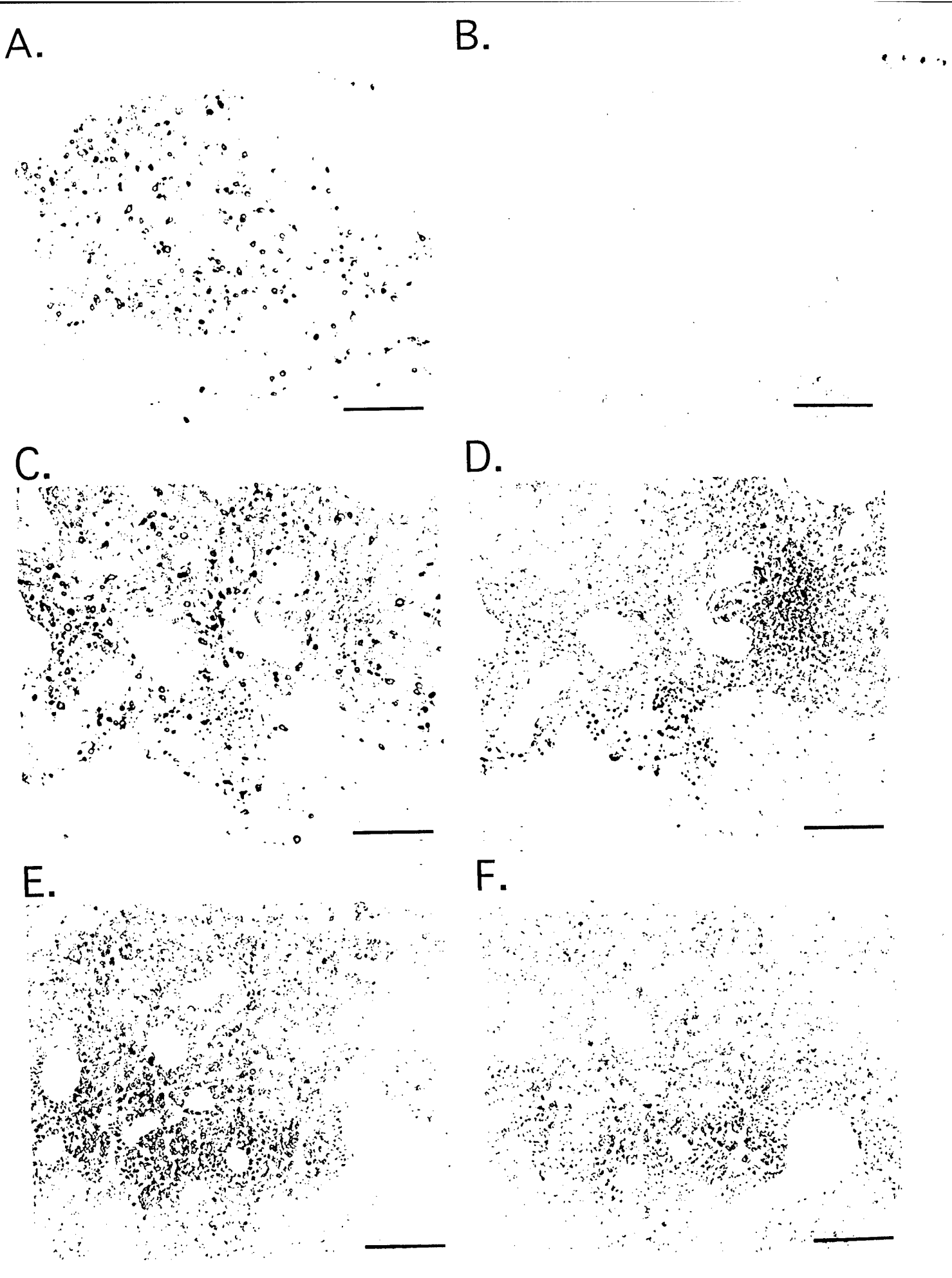
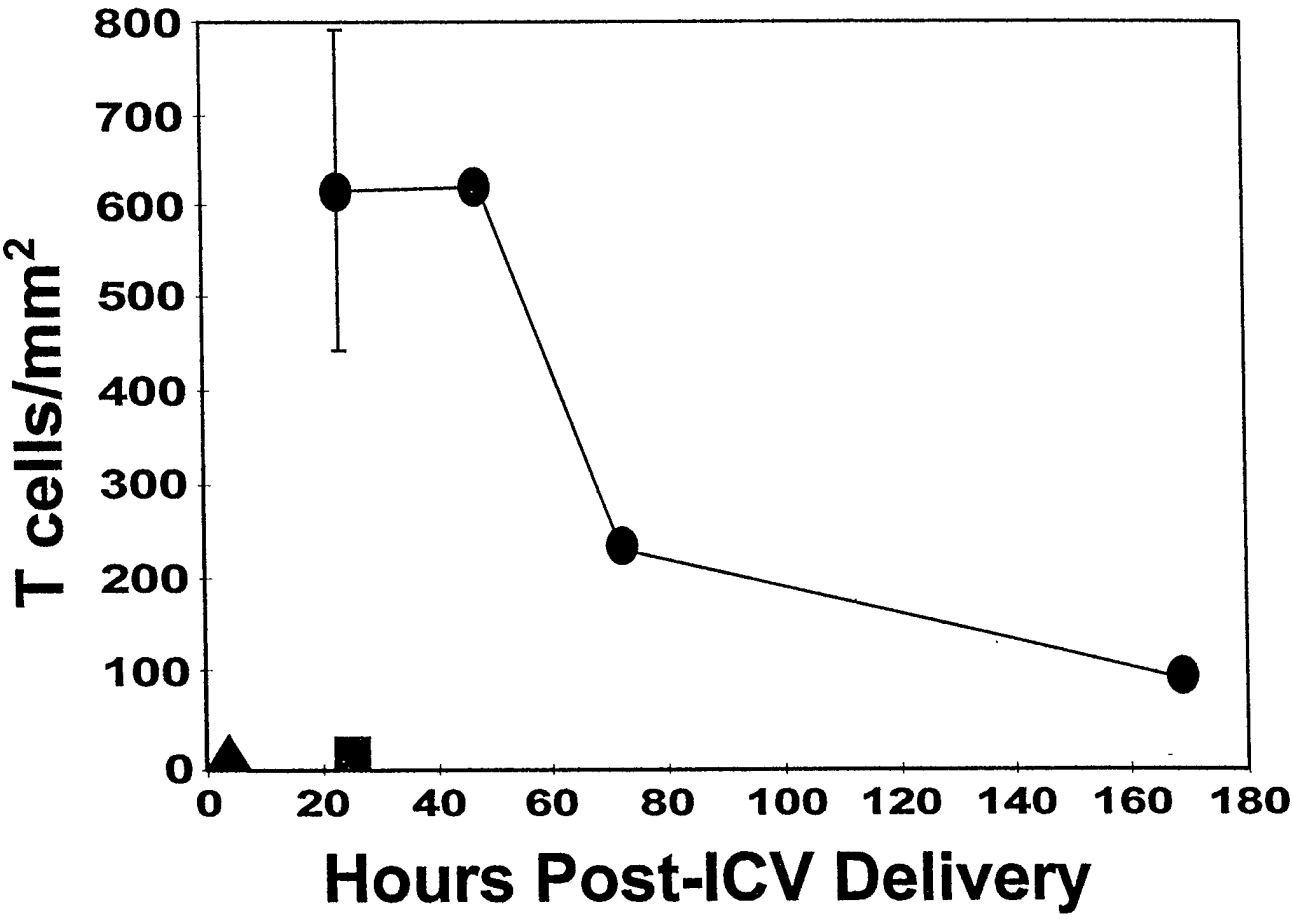
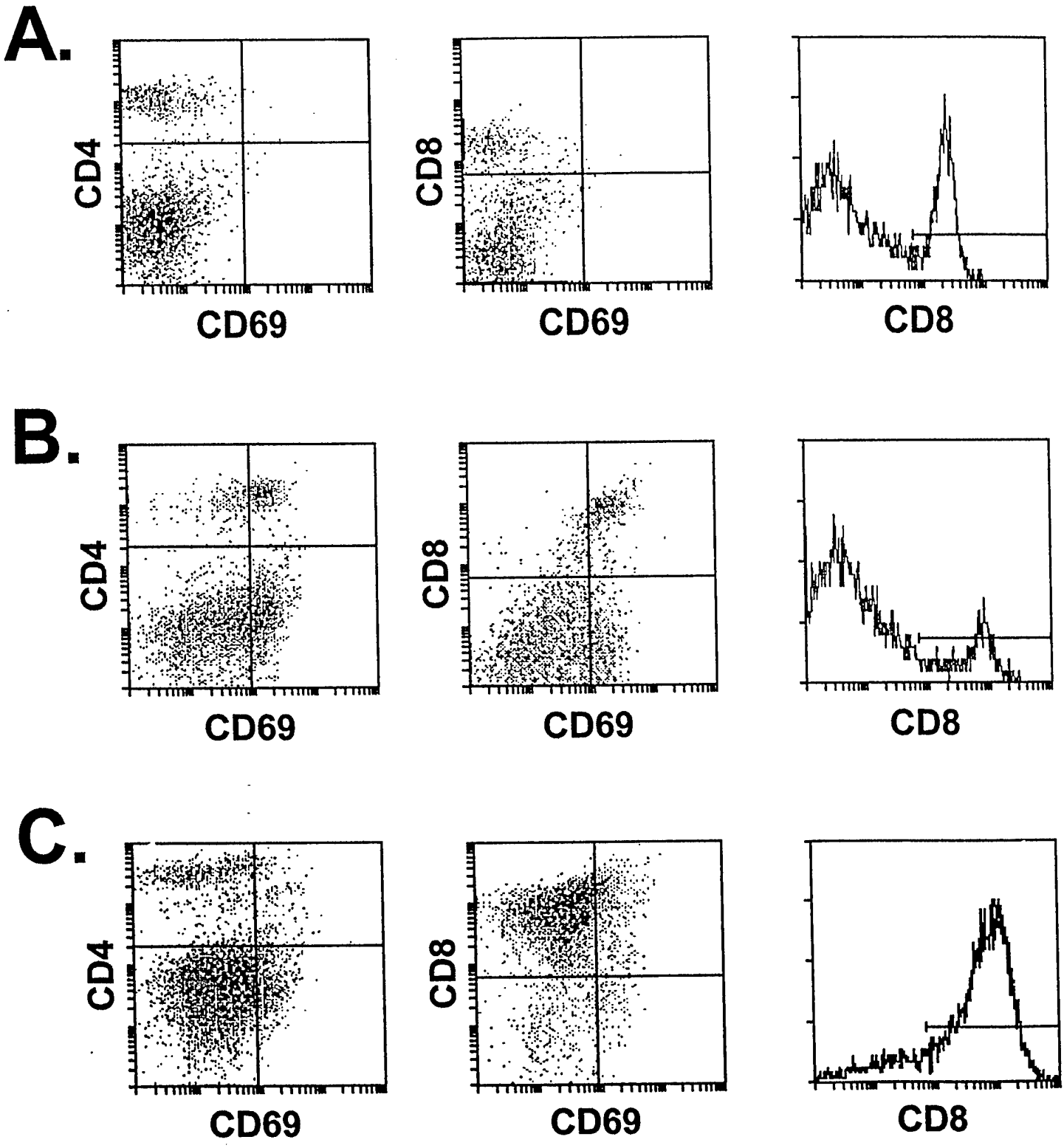
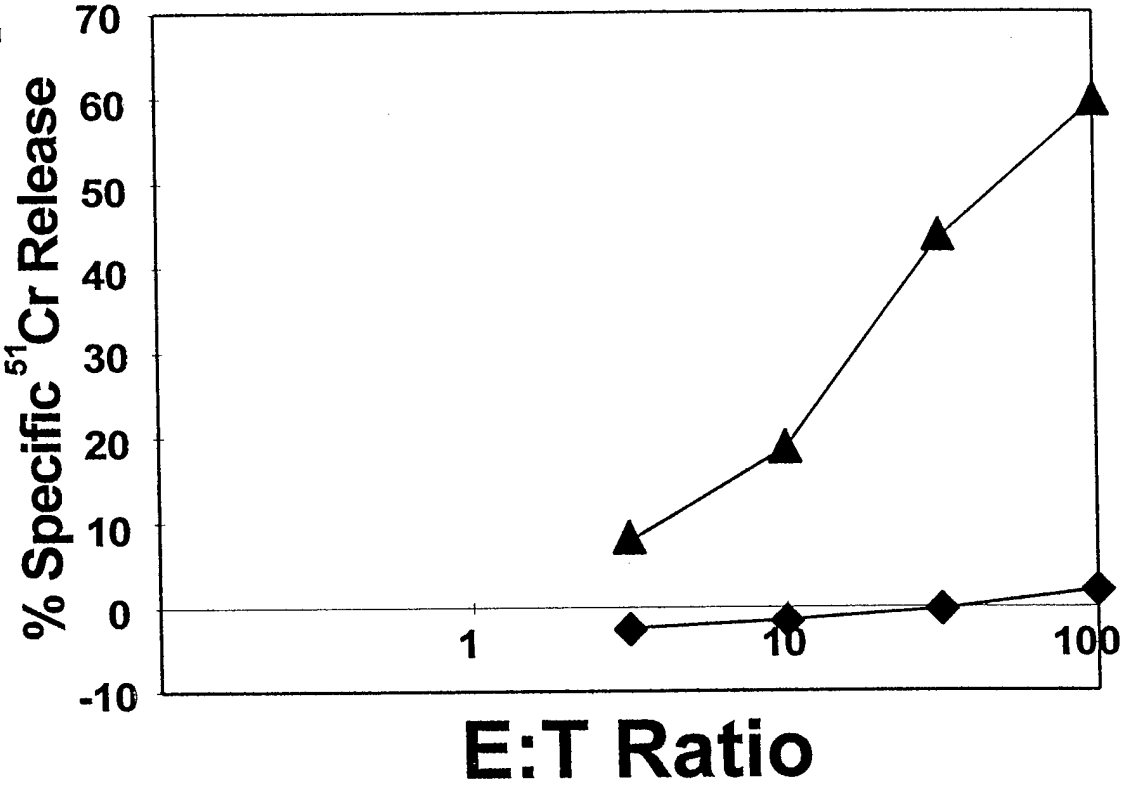
-5
% Specific 51Cr Release
1
10
100
E:T Ratio


A.
B.
C.
D.
E.
F.

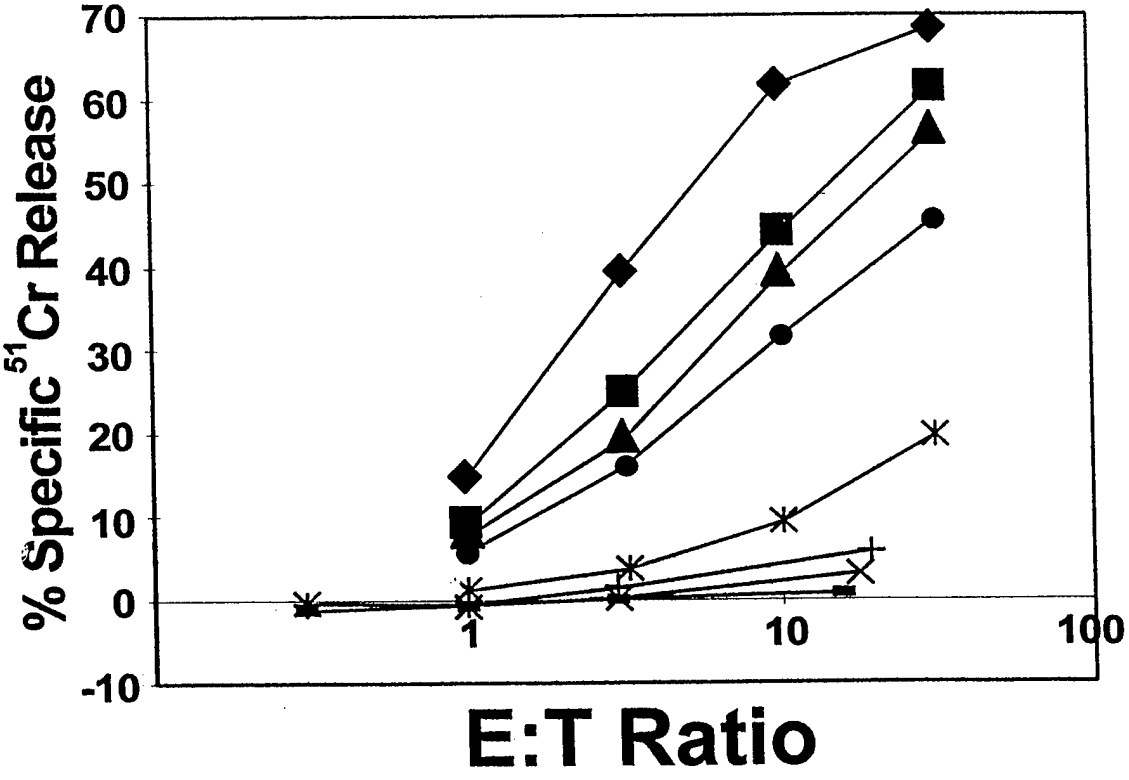

800
700
600
500
400
300
200
100
0
T cells/mm2
0
20
40
60
80
100
120
140
160
180
Hours Post-ICV Delivery


A.
CD4
CD69
CD8
CD69
CD8
B.
CD4
CD69
CD8
CD69
CD8
C.
CD4
CD69
CD8
CD69
CD8

A.


70
60
50
40
30
20
10
0
-10
% Specific 51Cr Release
1
10
100
E:T Ratio

B.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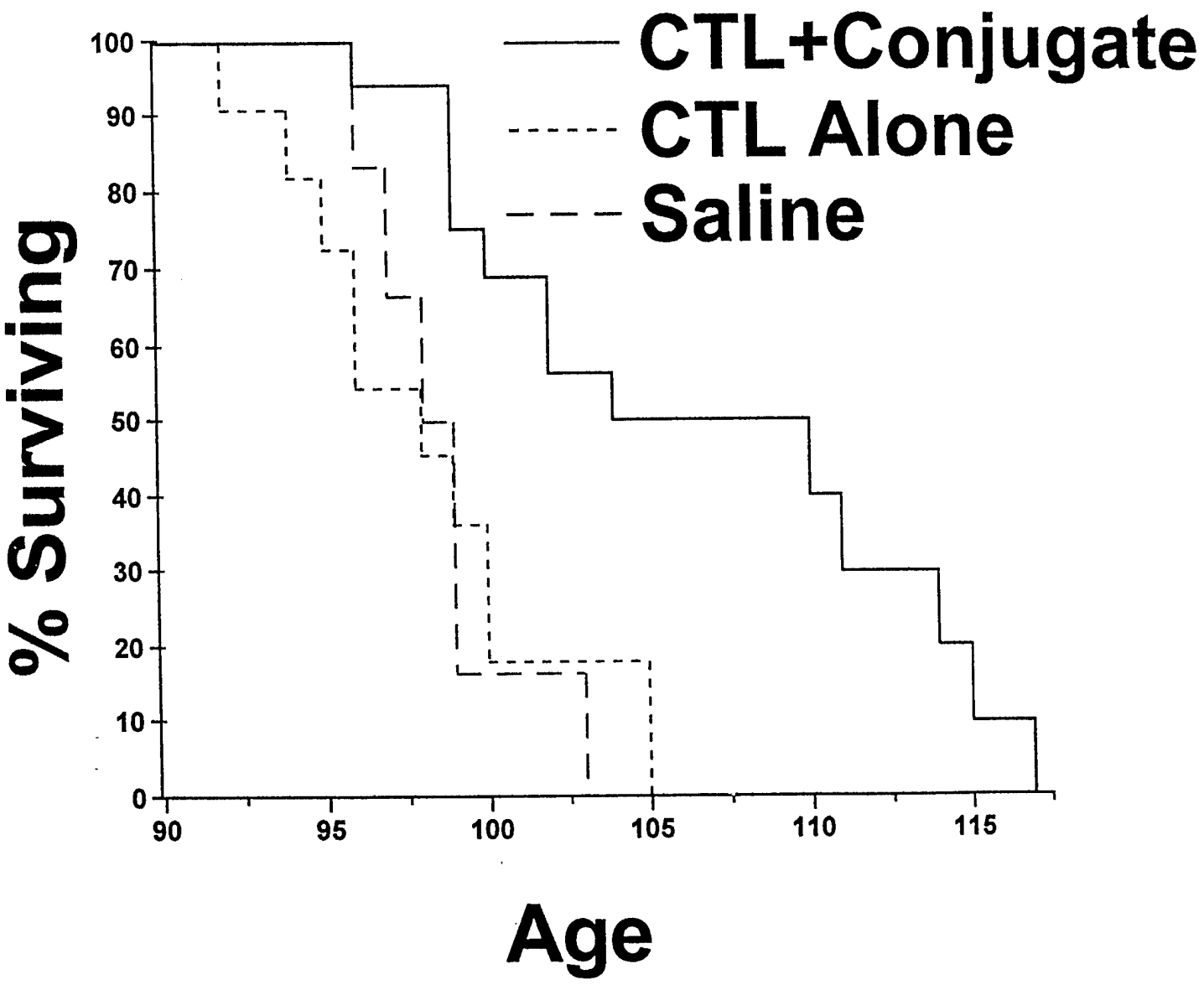
60
50
40
30
20
10
0
-10
% Specific 51Cr Release
1
10
100
E:T Ratio


CTL+Conjugate
CTL Alone
Saline
% Surviving
100
90
80
70
60
50
40
30
20
10
0
90
95
100
105
110
115
Age